Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/US05/008973

International filing date: 18 March 2005 (18.03.2005)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/554,538
Filing date: 19 March 2004 (19.03.2004)

Date of receipt at the International Bureau: 12 May 2005 (12.05.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

World Intellectual Property Organization (WIPO) - Geneva, Switzerland
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse

1315301

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

April 28, 2005

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE.

APPLICATION NUMBER: *60/554,538*
FILING DATE: *March 19, 2004*
RELATED PCT APPLICATION NUMBER: *PCT/US05/08973*

Certified by

Jon W. Dudas

Under Secretary of Commerce
for Intellectual Property
and Director of the United States
Patent and Trademark Office

Please type a plus sign (+) inside this box → [+]

PTO/SB/16 (5-03)
Approved for use through 4/30/2003. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Hyesook	Kim	Bloomfield Hills, Michigan
Alan A.	Dobkowski	Canton, Michigan
Raymond F.	Novak	Orchard Lake, Michigan
Brian B.	Haab	Jenison, Michigan

☐ *Additional inventors are being named on th____ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (280 characters max)

ANTIBODY AND PROTEIN MICROARRAYS: CHIP PRODUCTION, HYBRIDIZATION AND DATA INTERPRETATION

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

☐ Customer Number [ ] →

OR *Type Customer Number here*

Place Customer Number Bar Code Label here

☒ Firm *or* Individual Name	KOHN & ASSOCIATES, PLLC				
Address					
Address	30500 Northwestern Highway, Suite 410				
City	Farmington Hills	State	Michigan	ZIP	48334
Country	US	Telephone	(248) 539-5050	Fax	(248) 539-5055

ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages* 33 ☐ CD(s), Number

☐ Drawing(s) *Number of Sheets* ☒ Other (specify) Acknowledgement Postcard

☐ Application Data Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT *(check one)*

		FILING FEE AMOUNT ($)
☒	Applicant claims small entity status. See 37 CFR 1.27.	
☒	A check or money order is enclosed to cover the filing fees	
☒	The Director is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number 11-1449	$80.00
☐	Payment by credit card. Form PTO-2038 is attached.	

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

SIGNATURE [signature]

Date 3-19-04

TYPED or PRINTED NAME Amy E. Rinaldo

REGISTRATION NO. *(if appropriate)* 45,791

Docket Number: 3087.00014

TELEPHONE (248) 539-5050

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

If you need asssitance in completing the form, call 1-800-PTO-9199 and select option 2.

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

Express Mail: EV407003957US
Attorney Docket: 3087.00014

Provisional Patent Application

ANTIBODY AND PROTEIN MICROARRAYS: CHIP PRODUCTION, HYBRIDIZATION AND DATA INTERPRETATION

ABSTRACT

A method to produce antibody chips using monoclonal and polyclonal antibodies is disclosed. The method further includes producing antibody chips with anti-sera or ascites fluids without purification. A method to improve quality of antibody or protein microarrays by hybridizing probes with a blocking buffer that contains bovine serum albumin, purified milk proteins and a detergent is disclosed. The quality of microarrays can be further improved by hybridizing the antibody or protein chips with probes of high protein levels and with high hybridization volume. The invention also teaches the production of a drug-metabolizing enzyme antibody chips that can be utilized to screen expression levels of cytochromes P450 (CYPs), co-enzymes for CYP activity, apoptosis-related proteins, intracellular protein degradation proteins, Phase II drug-metabolizing enzymes, anti-oxidant proteins, and oxidant-stress proteins along with intracellular kinases and signaling kinases and phosphatases.

GOVERNMENT SUPPORT

Research in this application was supported in part by a contract from National Institute of Environmental Health Sciences (N43 ES 35506). The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

1. FIELD OF THE INVENTION

This invention relates to methods to produce antibody chips using monoclonal and polyclonal antibodies is disclosed. The method further included producing antibody chips with anti-sera or ascites fluids without purification. A method to improve quality of antibody or protein microarrays by hybridizing probes with a blocking buffer that contains bovine serum albumin, purified milk proteins and a detergent is disclosed. The quality of microarrays can be further improved by hybridizing the

antibody or protein chips with probes of high protein levels in high hybridization volume. This invention also include production of a drug-metabolizing enzyme antibody chips.

2. DESCRIPTION OF RELATED ART

Recently, user-friendly antibody microarrays were developed, however the antibodies that were used for the arrays were limited to monoclonal antibodies (1-3). This emerging technology needs to be improved to increase sensitivity, to lower background problems, and to accommodate polyclonal antibodies.

Previously the chips were hybridized for 2 hours at 4°C with ~15 µg of probes for each dye (in total ~30 µg/25 µl) with milk proteins (1), for 2 hours at room temperature with ~50 µg of probes for each dye (in total ~100 µg/80 µl) with milk proteins (3) or for 1 hour at room temperature with ~100 µg of probes for each dye (in total ~200 µg/80 µl) with 0.5% Tween-20 (2). This did not create a microarray that overcomes the problems detailed above.

DETAILED DESCRIPTION OF THE INVENTION

Generally, the present invention provides a method for producing antibody chips using monoclonal and polyclonal antibodies. The method further provides producing antibody chips with anti-sera or ascites fluids without purification. A method to improve quality of antibody or protein microarrays by hybridizing probes with a blocking buffer that contains bovine serum albumin, purified milk proteins, and a detergent is provided.

The quality of microarrays can be further improved by hybridizing the antibody or protein chips with probes of high protein levels and with high hybridization volume. The invention also teaches the production of a drug-metabolizing enzyme antibody chips that can be utilized to screen expression levels of cytochromes P450 (CYPs), co-enzymes for CYP activity, apoptosis-related proteins, intracellular protein degradation proteins, Phase II drug-metabolizing enzymes, anti-oxidant proteins, and oxidant-stress proteins along with intracellular kinases and signaling kinases and phosphatases.

In contrast to the prior art methods, Applicants obtained excellent results without non-specific binding to blank areas or a high background problem with hybridization of the chips with longer hybridization time (4 hours) at room temperature, higher volume of hybridization solution, higher protein levels and addition of milk proteins, BSA and a detergent (Tween-20). The hybridization solutions, including various blocking proteins and a detergent, prevented non-specific binding of dye conjugated proteins to additional proteins spotted on chips when anti-serum or ascites with a high protein level was spotted.

For example, polyclonal antibodies for human CYP3A4 were produced and characterized by Applicants and determined to be specific for rat CYP3A (CYP3A1 and 3A2). The CYP3A antibody spotted on the chip showed strong signals with phenobarbital-treated rat liver samples (2.6- and 2.1-fold increase with 50 and 500 µg/ml IgG spots, respectively). In addition to the above antibody, polyclonal antibodies as a form of IgG or anti-sera were spotted on the chip. So far, any antibody chip produced with polyclonal antibodies has not been reported. Thus, the results obtained at the laboratory demonstrated for the first time that polyclonal antibodies also were suitable for antibody chip production.

In addition, Applicants have demonstrated that anti-sera or ascites (without IgG purification, thus containing high levels of proteins) could be spotted on the chip for a successful antibody microarray analysis.

A detailed description of the invention is set forth in Appendix A included herewith and incorporated by reference in its entirety.

Literature Cited

1. Haab, B. B., Dunham, M. J., Brown, P. O. Genome Biol. 2, Research 0004.1-0004.12 (2001).
2. Streekumar, A., Nyati, M. K., Varambally, S., Barrette, T. R., Ghosh, D., Lawrence, T. S. and Chinnaiyan, A. M. Cancer Res. 61, 7585-7593 (2001).
3. Miller, J. C., Zhou, H., Kwekel, J., Cavallo, R., Burke, J., Butler, E. B., Teh, B. S. and Haab, B. B. Proteomics 3, 56-63 (2003).

APPENDIX A

Personnel:

Detroit R&D, Inc.

7/1/2003-8/31/2003:	Hyesook Kim, Ph.D. (25.5% of effort)
10/1/2003-10/30/2003:	Hyesook Kim, Ph.D. (25.5% of effort)
8/1/2003-8/30/2003:	David Kaplan (8% effort)
10/1/2003-11/30/2003:	David Kaplan (42.5% effort)
11/1/2003-11/30/2003:	Jung-A Park, Ph.D. (85% effort)
12/1/2003-12/23/2003:	Jung-A Park, Ph.D. (22% effort)

Subcontractor: Wayne State University

7/1/2003-3/31/2004:	Alan A. Dombkowski, Ph.D. (8% effort)
7/1/2003-3/31/2004:	Amy Diakiw (13% effort)

Consultants: Alan A. Dombkowski, Ph.D. (1 day)
Raymond F. Novak, Ph.D. (1 day)
Brian B. Haab, Ph.D. (1 day)

Introduction

Recently, user-friendly antibody microarrays have been developed but antibodies used for the arrays were limited to monoclonal antibodies. This emerging technology needs to be improved to increase sensitivity, lower background problem and accommodate polyclonal antibodies.

During Phase I, we proposed to find the best conditions for antibody chip production using monoclonal and form-specific polyclonal antibodies to produce rat drug-metabolizing enzyme chips, produce rat reference proteins and perform array analyses with rat liver samples to explore the utility of the chips.

Detroit R&D successfully produced and commercialized a targeted array with form-specific antibodies of closely related drug-metabolizing enzymes (see **an attached flyer**). The kit developed by Detroit R&D will be an invaluable tool for toxicoproteomics.

The technology developed during Phase I including sample preparation, scanning and data interpretation skills will provide a solid foundation for Phase II study. A website, www.antibodychip.com, was obtained to facilitate communications with collaborators and customers.

Results and Discussion

Objective 1. Rat drug-metabolizing enzyme antibody chip production.

(a) Selection of monoclonal and polyclonal antibodies.

Specificity of ~40 antibodies for drug-metabolizing enzymes available at Detroit R&D have been characterized at laboratories of Detroit R&D or our collaborators. Additionally, ~40 antibodies of interest were obtained from various commercial sources (**Table 1**). The commercially available antibodies were characterized at the commercial source for the

specificity. One exception is the UDP-glucuronosyl transferase antibody which was produced by immunization of a rabbit with a synthetic human form 1A peptide. Cross-reactivity with rat UDP-glucuronosyl transferase 1A is not known.

Western blot analysis with hepatic microsome + cytosol proteins obtained from untreated rats and rats treated with phenobarbital (PB) revealed that the antibody for human UDP-glucuronosyl transferase 1A recognized a rat form (see **Fig. 6**).

(b) First antibody chip production with 10 monoclonal antibodies.

Prior to antibody chip production with ~80 antibodies, test chips was produced with 9 antibodies consisting of 7 cytochrome P450 (CYP) monoclonal antibodies (anti-rat CYP1A1, CYP1A1/1A2, CYP2B1, CYP2B1/2B2, CYP2C11, CYP3A1 and CYP3A2), monoclonal antibody for Flag proteins (a control for dye conjugation and hybridization) and monoclonal antibodies for G3PDH (a house keeping protein). The Flag proteins are spotted twice, thus 6 times on each chip. The CYP monoclonal antibodies are specific and bind strongly to their antigens (they are being marketed at Detroit R&D).

The antibodies were spotted on a nitrocellulose-based antibody chips (FAST™ PAK, Schleicher & Schuell) with 0 (PBS), 0.5, 5, 50 and 500 μg/ml antibody solution. Three blocks of antibodies (thus, triplicate for each concentration) were spotted at Genomics Technology Support Facility, Michigan State University, using a split pin technology (spotting size, 1 nl).

Cy5-conjugated anti-mouse IgG (500 μg/ml) was obtained from Biomeda Co. (Foster City, CA) and 1 μl of 0, 10-, 100-, 1000- and 100,000-fold diluted IgG solution was spotted on a polyacrylamide (Hydrogel™)-based antibody chips (PerkinElmer) and scanned by Agilent G2565AA two-laser microarray scanner. Any solution diluted higher than 100-fold failed to show a red signal (**Fig. 1**).

The monoclonal antibody FAST chip was blocked for 45 min at room temperature with TTBS solution. The chip was hybridized with 100 μl of 100-fold diluted Cy5-conjugated antibodies (0.5 μg IgG/100 μl TTBS) for 1 hr at room temperature. Washing was carried out with TTBS for 4 times. The chip was dried and imaging was carried out using GenePix 4000A (Axon Instruments, Inc., Foster city, CA). Cy5 signals were detected with spots coated with 500 μg of IgG. The Agilent scanner wasn't suitable for the FAST chip because nitrocellulose membrane coated on the chip blocked fluorescent light.

(c) Landmarks and internal controls for probe labeling efficiency.

An internal control serves as a landmark for orientation of the chip and as an internal control for probe labeling and hybridization efficiency.

Two candidates tried with the chips during the Phase I were (a) Met + Flag peptide and (b) poly-His (6-His) which were recognized by anti-Flag and anti-6-His, respectively. These peptide sequences to be used for fluorescent signal normalization and landmarks are not present in mammalian cells.

Antibodies for Flag peptides and 6-His were obtained from Sigma Co. and Covance Research Products, Inc., respectively. Flag peptides were obtained from Sigma Co. 6-His peptides were synthesized and cross-linked to BSA by C-terminal carboxyl group of His.

0.5, 1 and 2 μg of the Flag landmark protein and 25, 50 and 100 μg of 6-His-conjugated BSA were added to proteins (250, 500 and 1000 μg, respectively) for both Cy3 and Cy5 labeling. The Flag landmark was used for microarry analyses with 2nd and 3rd antibody chips and both Flag and 6-His landmarks were used with 4th antibody chips.

A normalization factor (NF) obtained by the Flag landmark was ~1 as shown in the 3rd antibody chip experiments (**Fig. 3**). This result demonstrated that the NF obtained by landmark was same as the NF from total Cy3 or Cy5 bound to antibodies on the chip.

(d) Second antibody chip production with 76 monoclonal and polyclonal antibodies.

Second antibody chips were produced by spotting ~80 antibodies (~30 monoclonal and ~50 polyclonal antibodies) for drug-metabolizing enzymes and proteins with related functions and house-keeping proteins (see **Table 1**). The antibody solutions with increasing concentration of 0 (PBS), 0.5, 5, 50 and 500 μg/ml were spotted on both polyacrylamide (Hydrogel)- and nitrocellulose (FAST)-based antibody chips at the Michigan Life Sciences Corridor (MLSC) Proteomics facility at Van Andel Institute. The top block of antibodies with duplicate spotting for each concentration was repeated on the bottom block (thus, quadruplicate for each concentration).

The amount of IgG spotted on the chip was determined by protein levels determined at Detroit R&D, collaborators or at commercial sources. However, for anti-serum or ascites, IgG levels were estimated to be 1 mg/5 ml anti-serum or ascites. Total protein levels of anti-serum was estimated to be ~60 mg/ml. According to this estimation, 500 μg/ml solution contained ~6 mg/ml. Protein levels determined at Detroit R&D for mEH, CYP3A, NADPH-P450 reductase and CYP2D anti-sera were 75, 38, 45 and 45 mg/ml, respectively.

Quality control carried out by hybridizing the chip with anti-mouse, anti-rabbit or anti-goat IgG. Signal levels of antisera or ascites with 50 and 500 μg/ml spots were similar to the levels obtained with use of isolated IgG spots. This result demonstrated that estimation of IgG levels of the anti-sera or ascites was correct.

Spotting efficiency was assessed by hybridizing the spotted antibodies with 5 μg (1 ml) Cy5-conjugated antibodies for mouse, rabbit and goat IgGs (Biomeda Co.). Both chips showed cross-reactivity with the probes at 50 and 500 μg/ml antibodies (spotting volume, 1 nl). Hydrogel chips showed extremely high signals with the Cy5-conjugated probes. Images obtained with probes for rabbit IgG hybridized with antibodies spotted on Hydrogel and FAST slide are shown in **Fig. 2**. The fuzzy spots with the Hydrogel and white spots with the FAST slide are due to too strong Cy5 signals.

These results demonstrated that Hydrogel retained higher levels of spotted antibodies or retained the native antibody structure compared to the planar surface of the nitrocellulose-based FAST slide.

Objective 2. Production of rat reference proteins.

(a) Production of reference mixes for microarray analysis of PB treatment.

It has been reported previously that high protein levels in microarray hybridization solution caused a high background problem (**1**). So far maximum sample protein levels used in hybridization solution are ~200 μg (total hybridization volume, 80 μl) (**2**).

However, Detroit R&D successfully developed microarray chips and a hybridization protocol which uses ~7 mg proteins (2 mg of sample proteins + 0.75 mg BSA + ~4 mg purified milk proteins, hybridization volume, 750 μl). Non-specific binding and background problem were solved by bigger hybridization solution volume and by addition of more efficient blocking solution (BSA and purified milk proteins and detergent) for hybridization.

Previouosly, researchers tried to produce reference protein mix from purified proteins. However, the newly developed hybridization protocol enabled us to use a reference mix obtained from mixtures of microsomes and cytosol because additional proteins in the hybridization solution will not increase non-specific binding or induce a background problem. Still every protein to be quantitated by the antibody microarray has to be present in the reference mixture in a level higher than the signal cut-off (e.g. by GeneSpring software, 10 is a cut-off levels. Signal strength of majority of proteins used for microarray analyses using 3rd antibody chips was ~100 with 500 μg antibody spots by the GeneSpring analyses (**Fig. 3**). In the 500 μg antibody spotting analysis, only 5 proteins showed signals lower than 20 in both control and PB treated samples. They are inducible NO synthase (iNOS), prostaglandin E_2 synthase, CYP2E1, iron regulatory protein-1 (IRP-1) and guanylyl cyclase beta 1.

The mean signal level of the Flag proteins at 500 μg/ml antibody spots with GeneSpring anlyses (16 data points x 3 set) was 2663 $\pm$ 569. This signal was obtained by spiking 2 μg Flag protein to 1 mg rat protein sample prior to dye conjugation. Thus, it was estimated that ~75 ng protein/1 mg sample protein results in increase of ~100 signal with GeneSpring analysis.

Use of reference proteins for antibody microarrays is advantageous for an inter-laboratory comparison of the array results. However, unless the same reference mix is used, a comparison of the data is misleading. The reference mixes were produced in large quantity and stored as an aliquot at -80°C in a single use portion.

Our strategy for suitable reference mix production is that spiking 75 μg of the 5 low abundant proteins to 1 mg of the reference mix (microsomes + cytosol obtained from 2 untreated rats and 2 PB treated rats).

(b) Purification of microsomal and cytosolic proteins using protein chip (first protein chip production).

We developed a procedure that facilitates isolation of a protein. Hepatic cytosolic proteins obtained from rats after PB treatment were separated by a gel filtration chromatography. Approximately 400 fractions are collected. The fraction was transferred to a 384-well plate and spotted on nitrocellulose-based (FAST) chip using a split pin technology at Genomics facility, Michigan State University. The chip will be hybridized with an IgG of interest and washed followed by secondary hybridization of the chip with a Cy3- or Cy5-conjugated secondary IgG. Spots with a signal will be used to identify fractions containing proteins that bind the IgG.

Hepatic microsomal proteins obtained from rats after PB treatment were separated by an ion exchange chromatography. Approximately 400 fractions are collected. These fractions will be spotted and microsomal proteins of interest will be identified. CYPs are very closely related proteins. Further fractionation of the identified fraction will be carried out.

Objective 3. Exploration of utility of the antibody chips.

(a) Quality control of drug-metabolizing enzyme antibody chips (3rd antibody chip production).

Both polyacrylamide (Hydrogel)- and nitrocellulose (FAST)-based antibody chips were produced (2nd chip production) and spotting and hybridization efficiency of the chips were compared as described in Objective 1. Hybridization of the Hydrogel-based antibody chip with anti-mouse, anti-rabbit or anti-goat IgG showed fluorescent signals stronger than the signals obtained with the FAST chip.

Thus, 3rd antibody chips with 0.5, 5, 50 and 500 μl/ml antibody concentrations (1 nl per spot) were produced at the MLSC Proteomics facility at Van Andel Institute with Hydrogel chips and a quality control for spotting was carried out by hybridizing the chip with 1.25 μg Cy3-conjugated anti-rabbit IgG mixed with 2.5 μg Cy5-conjugated anti-mouse IgG (total volume, 1 ml). Hybridization and washing was carried out as described in Objective 1. In both dyes, spots with 0.5 and 50 μg/ml antibodies showed poor fluorescent signals whereas 50 and 500 μg/ml showed very strong signals (**Fig. 4, "Quality Control"**). Majority of the 50 and 500 μg IgG spots were round and even.

(b) Hybridization of the 3rd antibody chips (Hydrogel) with a direct design.

Using the 3rd antibody chips, microarray analyses were carried out with liver microsomes + cytosol obtained from 2 untreated rat and 2 rats after treatment with PB (100 mg/kg/day for 3 days). Two sets of array analyses were carried out using a direct design. Control and PB-treated protein samples were labeled with both Cy3 and Cy5 used for fluor swapping.

First set:

1. Cy3 rat #1 control **(Control 1)** vs. Cy5 rat #2 PB **(PB1)** microsomes/cytosol
2. Cy5 rat #1 control **(Control 1)** vs. Cy3 rat #2 PB **(PB1)** microsomes/cytosol

Second set:

3. Cy3 rat #3 control **(Control 2)** vs. Cy5 rat #4 PB **(PB2)** microsomes/cytosol
4. Cy5 rat #3control **(Control 2)** vs. Cy3 rat #4 PB **(PB2)** microsomes/cytosol

The Hydrogel chips were blocked overnight at 4°C with 1% BSA/PBS blocking solution as previously described by Haab et al. (**1**). The rat hepatic proteins were dialyzed with 10 kDa molecular cut-off membrane and analyzed for protein concentration. An internal control protein, Met-Flag fusion proteins of *E. coli* bacterial alkaline phosphatase recognized by anti-Flag (Sigma Co.) was spiked to both untreated and PB-treated samples prior to Cy3 or Cy5 conjugation.

The rat proteins (1 mg) with bacterial Flag protein (2 μg) were mixed and cross-linked with NHS-ester activated Cy3 or Cy5 (Amersham Biotech) (0.2 mg) according to the manufacturer's instructions. The probes were washed 3 times with 500 μl PBS and concentrated to 40 μl using microconcentrator spin columns (Amicon, 10 kDa cutt-off). As described above for Control 1/PB 1 set (first set) and control 2/PB 2 set (second set), hybridization of the probes with dye swapping (in total, 4 hybridizations, 16 data points for each IgG concentration) was carried out in PBS with 0.1% (0.75 mg) BSA, 0.01% Tween-20 and 3% (22 mg or 4 mg after purification) milk blocking proteins (total volume, 750 μl). The hybridization solution contained 2 mg of rat liver proteins. The level of milk proteins added to the solution was 4 mg after extensive purification (about 20% of 22 mg of the original 3% milk solution). A representative image is shown in **Fig. 4 "Experiment"**.

First hybridization of the chip with 250 μg for each probe (total 500 μg proteins with hybridization volume of 128 μl) was not successful due to faint signals of the hybridized spots. The protein level increased to 500 μg (total 1 mg proteins with hybridization volume of 238 μl) and to 1 mg (total 2 mg proteins with hybridization volume of 750 μl) improved quality of array images. Hybridization of the chip with 2 mg sample proteins (in total, ~7 mg proteins/750 μl) showed dark back ground lines. This problem was corrected by filtering milk proteins with a Glass Microfibre filter (Whatman) after dialyzing the milk proteins against PBS using a 10 kDa molecular cut-off membrane (**Fig. 5**).

Previously the chips were hybridized for 2 hr at 4°C with ~15 μg of probes for each dye (in total ~30 μg/25 μl) with milk proteins (**1**), for 2 hr at room temperature with ~50 μg of probes for each dye (in total ~100 μg/80 μl) with milk proteins (**3**) or for 1 hr at room temperature with ~100 μg of probes for each dye (in total ~200 μg/80 μl) with 0.5% Tween-20 (**2**)

In contrast, we obtained excellent results without non-specific binding to blank areas or a high background problem with hybridization of the chips with longer hybridization time (4 hr) at room temperature, higher volume of hybridization solution, higher protein levels and addition of milk proteins, BSA and a detergent (Tween-20).

The hybridization solutions with various blocking proteins and a detergent prevented non-specific binding of dye conjugated proteins to additional proteins spotted on the chips when anti-serum or ascites fluids with a high protein level was spotted.

(c) Quantitation and Bioinfomatics.

After hybridization, the chips were dried and images (4 images, 4 data points per image, in total, 16 data points) were scanned at 532 nm (green) and 635 nm (red) and pseudo-colored images were obtained using GenePix 4000A (Axon Instruments). A spot design file was produced from a spreadsheet prepared at our laboratory to identify location of each protein in the 384-well plate. Signal intensity of each target protein was quantitated with Genepix software using the spotting file which contained identity of each protein and its location in the 384-well plate.

The GenePix data file was incorporated into GeneSpring microarray clustering and statistical analysis (Silicon Genetics) and normalization of expression levels was carried out using total Cy3 and Cy5 signals.

The internal control protein for Cy3 and Cy5 conjugation and hybridization, Met-Flag fusion proteins of *E. coli* bacterial alkaline phosphatase, were spotted in 0.5, 5, 50 and 500 μg/ml solutions duplicate for 3 times in each block (top and bottom blocks). Thus each concentration has 16 data points (8 data points from Control 1/PB1 and 8 data points from Control 2/PB 2) of 3 sets. The fold increase of the 3 sets of flag proteins was 1.04-, 1.04- and 0.93-fold (mean, 1.00 ± 0.06) with 500 μg/ml spots and 1.15-, 0.88- and 0.82-fold (mean, 0.95 ± 0.18) in 50 μg/ml IgG spotting. If the number is lower than 1, expression levels of the protein decreased by PB treatment. This result confirmed that, with 50 and 500 μg/ml IgG spots, dye cross-linking and hybridization effects were correctly normalized by the GenePix software.

An interesting observation is that signal levels of each Flag proteins were somewhat different among 3 sets especially with 50 μg/ml spots (**data not shown**). Nonetheless, ratios obtained by direct competition of Cy3- or Cy5-conjugated control with Cy5- or Cy3-conjugated PB treated samples were very similar. This was due to variation in antibodies levels spotted on a chip. This result demonstrated that, with 2 fluors directly competing for a spot, a slight variation in spotting efficiency will not affect the outcome of a successful array analysis.

The signal cut-off level was set to 10 for GeneSpring analysis. Any consolidated data point lower than 10 for both control and PB treated samples were not recorded for fold change of the protein. At 0.5 and 5 μg/ml anti-Flag IgG spots, dye signals were lower than 10 in both Cy3 and Cy5 conjugated samples.

Out of 390 (78 antibodies X 5 concentrations) consolidated protein data (76 proteins + 2 additional Flag proteins with 5 various concentration of antibodies), 12 consolidated protein data showed up-regulated expression levels which were same or higher than 2-fold after PB treatment. They were identified as CYP2B1 (with 50 μg/ml spots), 30.6-fold; CYP2B1/2B2 (with 500 μg/ml spots), 9.7-fold; CYP 2B1 (with 500 μg/ml spots), 9.3-fold; mEH (with 5 μg/ml spots), 3.2-fold; mEH (with 50 μg/ml spots), 2.9-fold; CYP3A4 (with 50 μg/ml spots), 2.6-fold; calpain (with 5 μg/ml spots), 2.5-fold; calpain (with 50 μg/ml spots), 2.5-fold; COX-2 (with 500

μg/ml spots), 2.3-fold; 2D6 (with 500 μg/ml spots), 2.1-fold; CYP 3A4 (with 500 μg/ml spots), 2.1-fold; and COX-2 (with 50 μg/ml spots), 2.0-fold. Majority of 0.5 and 5 μg/ml antibody spots failed to show signal levels higher than 10 for either control or PB treated samples. All PBS spots (negatives) also failed to appear except for I contaminated well.

GeneSpring analysis of 500 μg/ml antibody spots are shown in **Fig. 3**. The middle diagonal line is for a protein expression level that did not change after PB treatment, the top line is for 2-fold increase and the bottom line is for 2-fold decrease.

Internal control, Flag proteins (3 sets) (2 μg), spiked to 1 mg of sample, were close to the middle line as expected and showed high signal values (mean value, 2663). GST alpha and antioxidant-like protein 1 also showed high expression levels without any change.

CYP2B1 and CYP2B1/2B2 spots were at above the 2-fold increase line with 9.3- and 9.7-fold increase, respectively, after PB treatment. CYP2B1 has been reported to be a primary protein that increased after PB treatment. An interesting observation is that both CYP 2B1 IgG and CYP2B1/2B2 IgG bound with CYP2B1 proteins.

All data obtained from 500 μg/ml antibody spots produced reliable data (signals higher than 20 in both control and PB treated samples) except for iNOS, prostaglandin E_2 synthase, CYP2E1, IRP-1 and Guanylyl Cyclase beta 1.

IRP-1 is a house keeping protein. The low signal may be due to weak binding of the antibody to IRP-1. Since the antibody chips contained 4 other housekeeping proteins, the antibody for IRP-1 protein was deleted for 4th antibody chip production. Anti-CYP2E1 was replaced with CYP2E1 IgG with higher affinity for 4th chip production.

Polyclonal antibodies for human CYP3A4 were produced and characterized at Detroit R&D to be specific for rat CYP3A (CYP3A1 and 3A2). The CYP3A antibody spotted on the chip showed strong signals with phenobarbital-treated rat liver samples (2.6- and 2.1-fold increase with 50 and 500 μg/ml IgG spots, respectively). In addition to the antibody, polyclonal antibodies as a form of IgG or anti-sera were spotted for this 3rd chip. So far, any antibody chip produced with polyclonal antibodies has not been reported. Thus, the results obtained at our laboratory demonstrated for the first time that polyclonal antibodies also were suitable for antibody chip production.

In addition, we demonstrated that anti-sera or ascites (without IgG purification, thus containing high levels of proteins) could be spotted on the chip for a successful antibody microarray analysis.

The Cy5 (red) signal of each spot on arrays decreases after scanning faster than the Cy3 signal. Each spot shown on the GeneSpring analysis was calculated from 9 Cy5 and 9 Cy3 data points after reversing ratios obtained by Cy3 (see **Fig. 3**). The Cy5 and Cy3 dye bias was found with the array analyses with 3rd chips (**data not shown**). The dye bias could be corrected by normalization of the data by the dye bias obtained with Flag proteins (**data not shown**).

(d) Pathways induced by PB treatment.

Pathway analysis of the data obtained by 3rd antibody chips are in progress using the GenMapp software (Univ. of California at San Francisco). This result will identify gene signaling and metabolic pathways changed by PB treatment.

(e) Verification of microarray analysis.

Protein levels of CYP2B1, CYP2B1/2B2, microsomal epoxide hydrorase (mEH), CYP3A4, calpain, COX-2 and 2D6 increased after PB treatment (see result obtained with 3rd chips). This

microarray result was obtained by combining results from Control 1 and PB 1 assays and Control 2 and PB 2 assays. Expression levels of CYP2B1, mEH and UDP-glucuronyltransferase (UGT) in Rat #1 (Control 1), Rat #2 (PB 1), Rat #3 (Control 2) and Rat #4 (PB 2) were verified by Western blot analyses of microsomes + cytosol (1:1) proteins (**Fig. 6**). Western blot analyses revealed that hepatic CYP2B1 and mEH proteins increased after treatment of Rat #4 with PB (PB 2) higher than the protein levels of Rat #2 (PB1) (**Fig. 6**).

(f) Production and hybridization of the 4th antibody chips (Hydrogel) with a reference design.

Fourth Hydrogel antibody chips were produced at the Genomics facility at Michigan State University using split pin technology. Data sheets to locate the position of each antibody were produced (**Fig. 7**). The electronic data sheets were used to produce a chip design file. The design file was used for quantitation of the arrays.

Five corrections were made with the 4th chips:

(1) An antibody for a house keeping protein, IRP-1, was deleted because 500 μg/ml IRP-1 antibody spot failed to show a signal higher than 20 with the array analyses using 3rd chips.

(2) CYP2E1 signal was very low (~20 with GeneSpring analysis) when CYP2E1 is expressed in untreated and PB treated rats. Several CYP2E1 IgG fractions at Detroit R&D were screened and previously spotted antibody was replaced with CYP2E1 antibody with stronger binding.

(3) IgG levels (or IgG levels translated to IgG expressed in anti-sera and ascites) was increased to 0.1, 0.3, 0.5 and 1 mg/ml. The 1 mg/ml anti-serum contains ~12 mg proteins/ml.

(4) In microarrays with 3rd chip, signals obtained from spots with increasing levels of proteins for each protein were scattered. This was advantageous because it could get rid of position related false positives. However, we concluded that, with 16 data points (4 data points per chip) which are strategically dispersed, would not have this problem. Thus, increasing levels of antibodies were spotted in a row. This will make a IgG dose-dependent signal increase visible.

(5) An additional internal control for dye conjugation and hybridization, 6-His, were spotted.

Quality control was carried out by hybridization of 2.5 μg Cy5-conjugated anti-mouse and 1.25 μg Cy3-conjugated anti-rabbit IgG (total volume, 1 ml). Reference proteins were produced as described in Objective 2 and array analyses will be carried out as follows.

1. Cy3 reference vs. Cy5 rat #3 control **(Control 2)** microsomes/cytosol
2. Cy5 reference vs. Cy3 rat #3 control **(Control 2)** microsomes/cytosol
3. Cy3 reference vs. Cy5 rat #4 PB **(PB2)** microsomes/cytosol
4. Cy5 reference vs. Cy3 rat #4 PB **(PB2)** microsomes/cytosol

(g) Whole cell extracts of rat liver primary cultured cells obtained after treatment with PB (1 mM).

Hepatocytes were isolated from the livers of male Sprague-Dawley rats (~300 g body weight) using collagenase perfusion, plated onto dishes covalently coated with Vitrogen (~11X10^6 cells/100 mm dish), and kept in modified Chee's medium supplemented with 0.1 μM insulin as previously described (**4,5**). Medium was changed at 4 hr after plating and every 24 hr

thereafter. At the 48 hr medium change, cells were treated with 1 mM PB for 48 hours. Hepatocyte extracts obtained from untreated and PB treated in triplicate were prepared as previously described (**5**). Microarray analyses with the extracts are in progress.

(h) Stability of antibodies spotted on chip.

Antibody chips were washed and dried as previously described by Haab et al. (**3**) and refrigerated in a desiccator. Antibodies are stable and usually their active sites are not easily damaged. Chips produced during Phase I were for an immediate use. However, for marketing, stability or a shelf-life of the antibody chip is a factor for a successful commercialization. During Phase II study, we will study stability of the antibody chips.

Literature Cited

1. Haab, B. B., Dunham, M. J., Brown, P. O. Genome Biol. 2, Research 0004.1-0004.12 (2001).
2. Streekumar, A., Nyati, M. K., Varambally, S., Barrette, T. R., Ghosh, D., Lawrence, T. S. and Chinnaiyan, A. M. Cancer Res. 61, 7585-7593 (2001).
3. Miller, J. C., Zhou, H., Kwekel, J., Cavallo, R., Burke, J., Butler, E. B., Teh, B. S. and Haab, B. B. Proteomics 3, 56-63 (2003).
4. Waxman, D. J., Morrissey, J. J., Naik, S., and Jauregui, J. O. Biochem. J. 271, 113-119 (1990).
5. Zanger, R. C., Woodcroft, K. J., Kocarek, T. A., and Novak, R. F. Drug Metab. Dispos. 23, 681-687 (1995).

Table 1. A list of antibodies spotted for rat drug-metabolizing enzyme microarrays

Antibody Available at Detroit R&D	Commercially Available		Antibody Available at Detroit R&D	Commercially Available
P450 Phase I Drug-Metabolizing Enzyme			**Phase II Drug-Metabolizing Enzyme**	
1A1			N-acetyltransferase form-I	
1A1/ 1A2			N-acetyltransferase form-II	
2A			GST-alpha	
3A1			GST-mu	
3A2			GST-pi	
3A23			Microsomal Epoxide Hydrolase	
3A1/3A2/3A23[a]				UDP-Glucuronosyltransferase
4A2			**Anti-Oxidant Protein**	
	1B1		Superoxide Dismutase	
2B1			Catalase	
2B1/2B2				Glutathione Peroxidase
2C11			**House Keeping Protein**	
2C23				Tubulinn
2D				Beta-Actin
2E1				G3PDH
2G1				Alpha-Actin
2J3/2J4				IRP-1
Co-Enzyme for CYP Activity			**Oxidative Stress Protein**	
Cytochrome b5				sPLA2
P450-NADPH-Reductase				iNO Synthase
Additional Phase II Drug-Metabolizing Enzyme				nNOSynthase
Carboxylesterase				eNOSynthase
Mitochondrial Protein			COX-1	
	Bax		COX-2	
	Bcl-2			5-Lipoxygenase
Cytochrome C				Thromboxane
	PBR			ProstaglandinE Synthase
Apoptosis-Related Protein			Calpain	
	Apaf1			NFkappaB
	Bad			PPAR (Peroxisome Proliferator-Activated Receptors)-Alpha
	Bak			P-Glycoprotein (P-gp)
	BID			TNF-alpha
	Caspase3			Heme Oxygenase-1
	Caspase8			Heme Oxygenase-2
	Caspase10			Guanylyl Cyclase a1
	FADD			Guanylyl Cyclase b1
	Fas			Beta Catenin
	p53			CEA(Carcinoembryonic Antigen)
	PARP			Antioxidant-like Protein 1
	TRAIL			
	Rb		**Internal Control**	
Intracellular Protein Degradation Protein				Flag
	Calpastatin			
	E1 Ubiquitin			

a: CYP3A4 which is specific for CYP3A subfamily

Fig. 1. Decrease of Cy5 signal strength of Cy5-conjugated anti-mouse IgG (500 µg/ml) after serial dilution

Dilution 0 10 100 1000 10000

Fig. 2. Comparison of antibody spotting efficiency of polyacrylamide (hydrogel)-based chips with nitrocellulose (FAST)-based chips.

The 0.5, 5, 50 and 500 µg/ml antibody solutions (~80 monoclonal and polyclonal antibodies for rat proteins) were spotted (spotting volume, 1 nl) in duplicate per 4 top blocks. The same 4 blocks were spotted on the bottom (4 data points per each concentration of antibodies). Cy5-conjugated antibodies (5 µg) for rabbit IgGs were hybridized with the chips. Scanning of the chips was carried out using GenePix 4000A (Axon Instruments). The fuzzy spots with the hydrogel and white spots with the FAST slide are due to too strong Cy5 signals.

Hydrogel (polyacrylamide based) FAST PAK (nitrocellulose based)

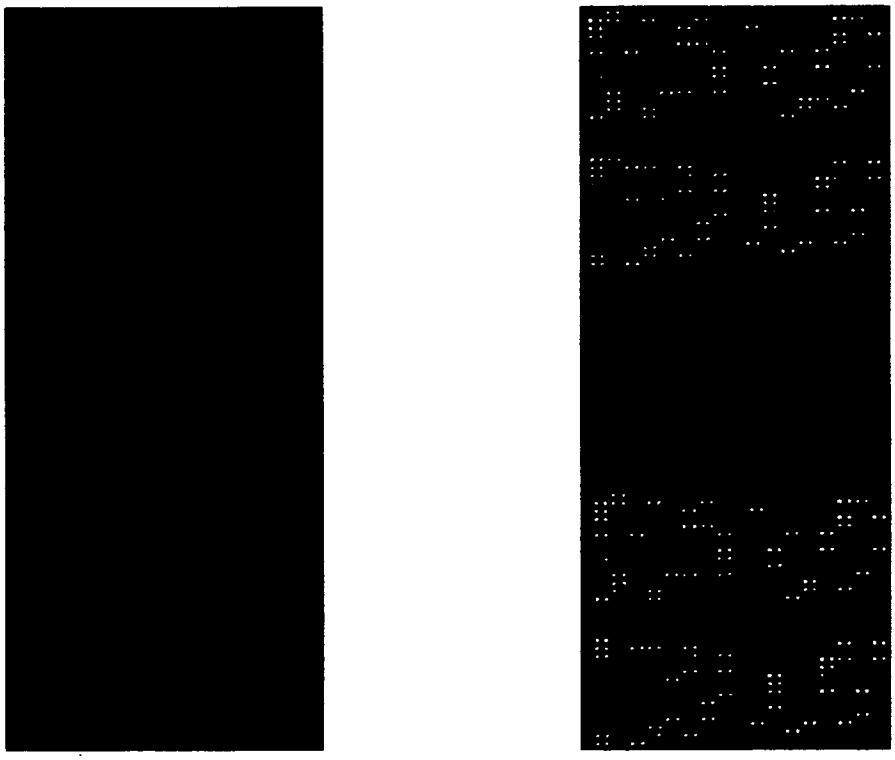


Fig. 3. Expression levels of rat hepatic proteins obtained from untreated rats and rats treated with phenobarbital. Consolidated data from 4 microarray analyses with 500 µg/ml antibody spotting (16 data points per spot). (AOP-1: Antioxidant-like Protein 1; iNOS: iNO Synthase and IRP-1:Iron Regulate Protein)

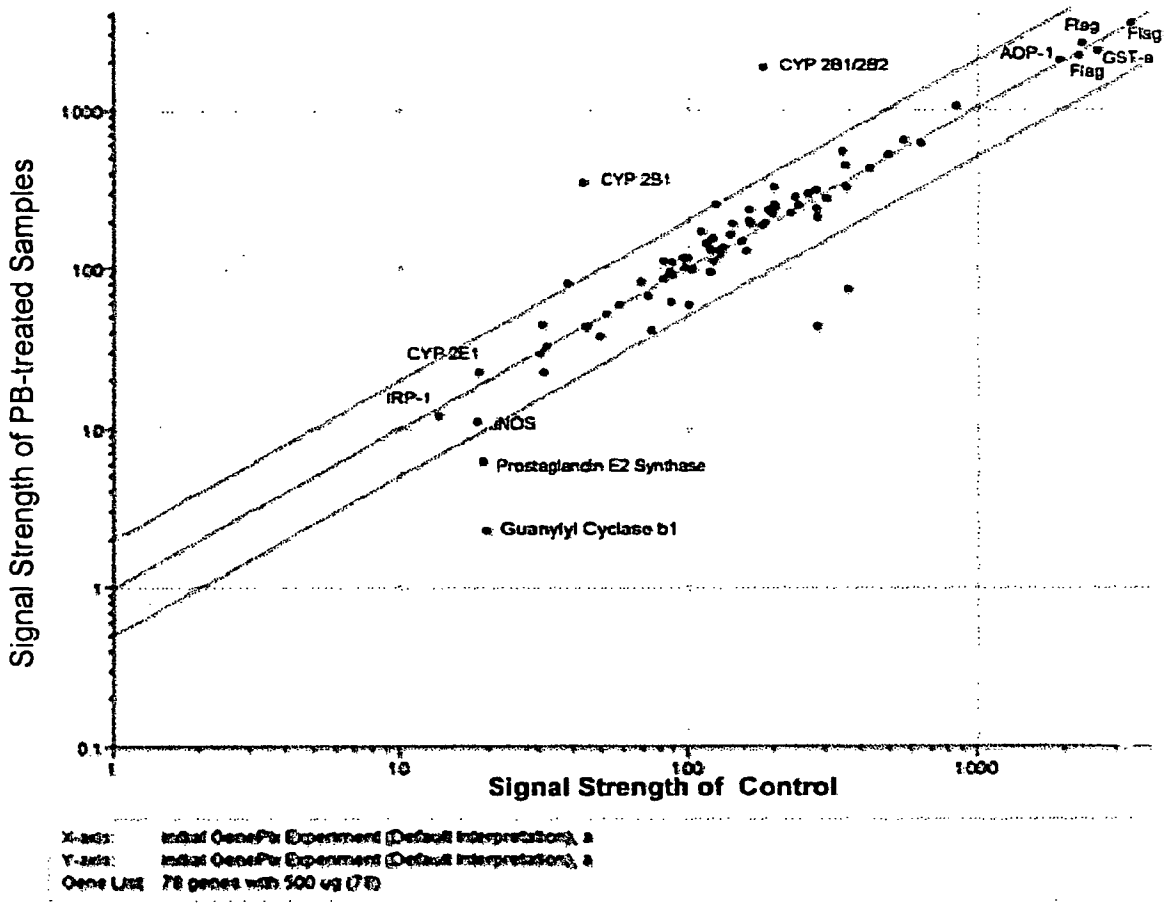


Fig. 4. Quality control of antibody chips using anti-mouse and anti-rabbit IgG and microarray analyses of rat hepatic proteins for phenobarbital treatment (a representative image is shown).

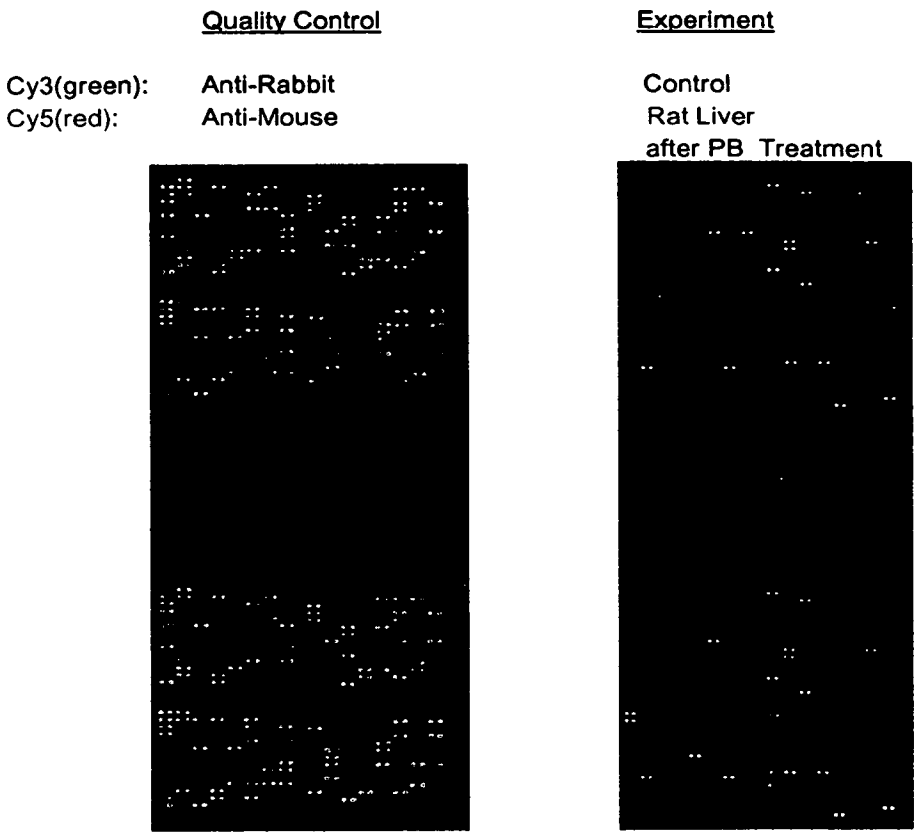


Fig. 5. Elimination of background black lines by filtration of milk proteins.

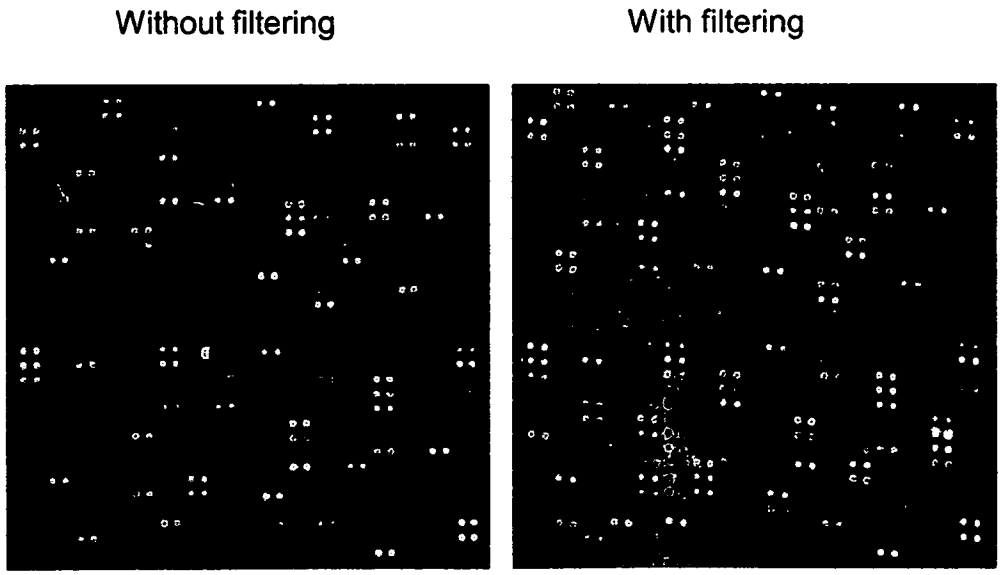


Fig. 6. Verification of microarray results by Western blot analyses in non-denaturing condition.

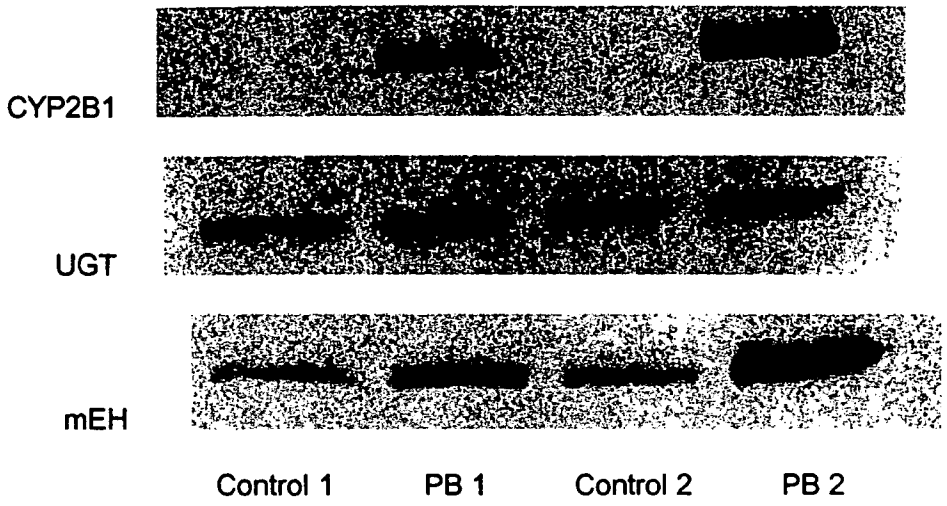
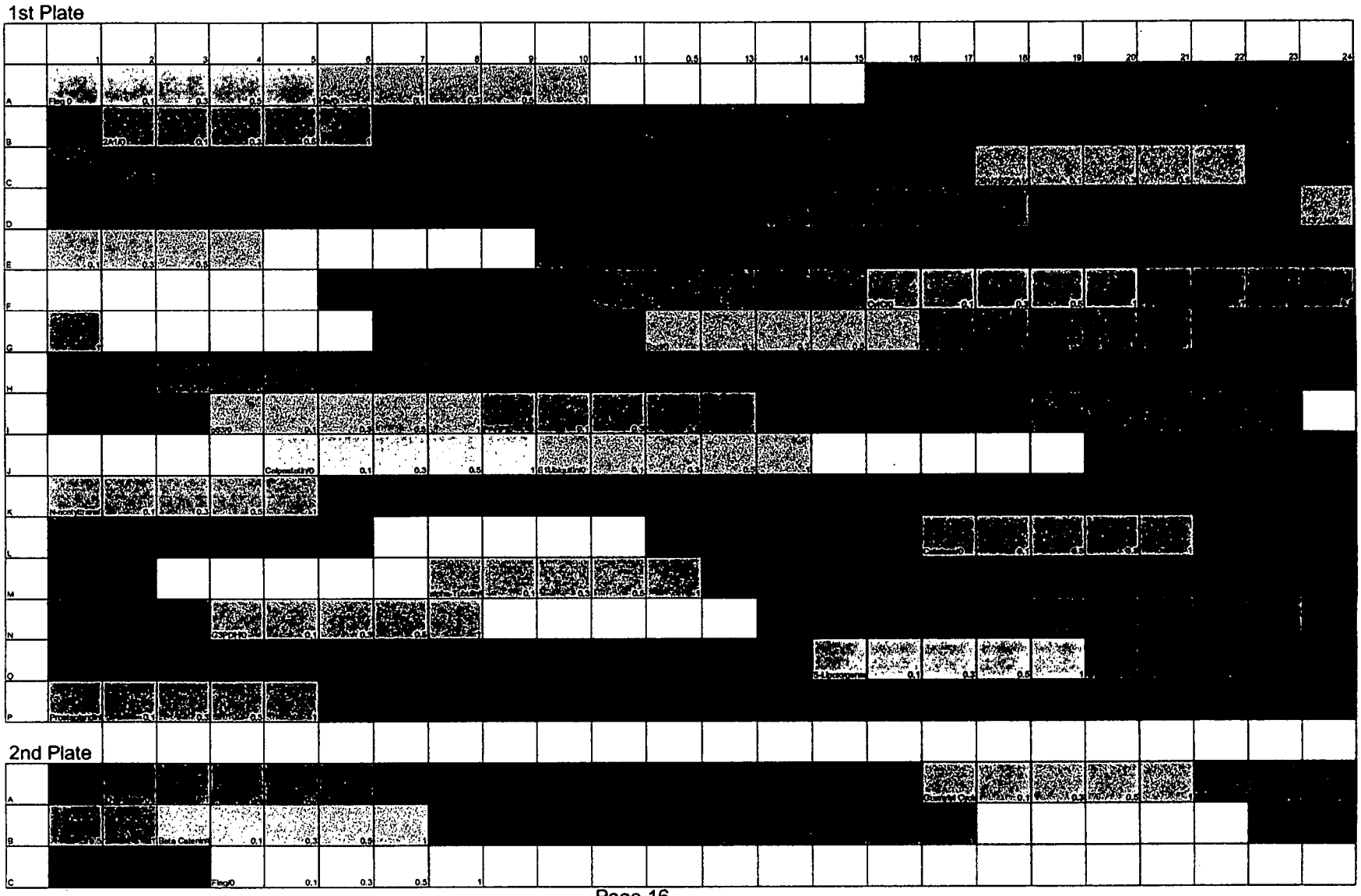
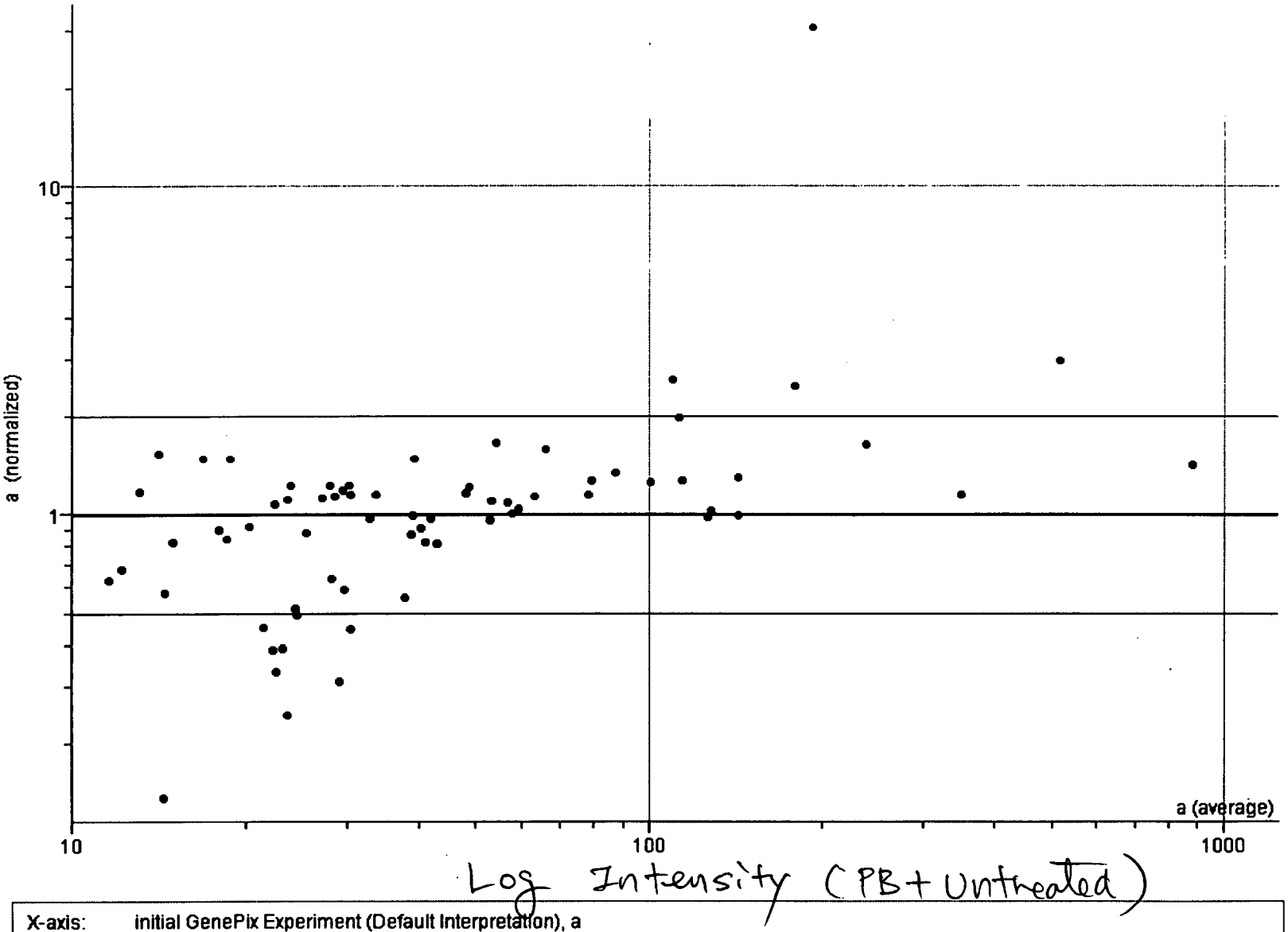


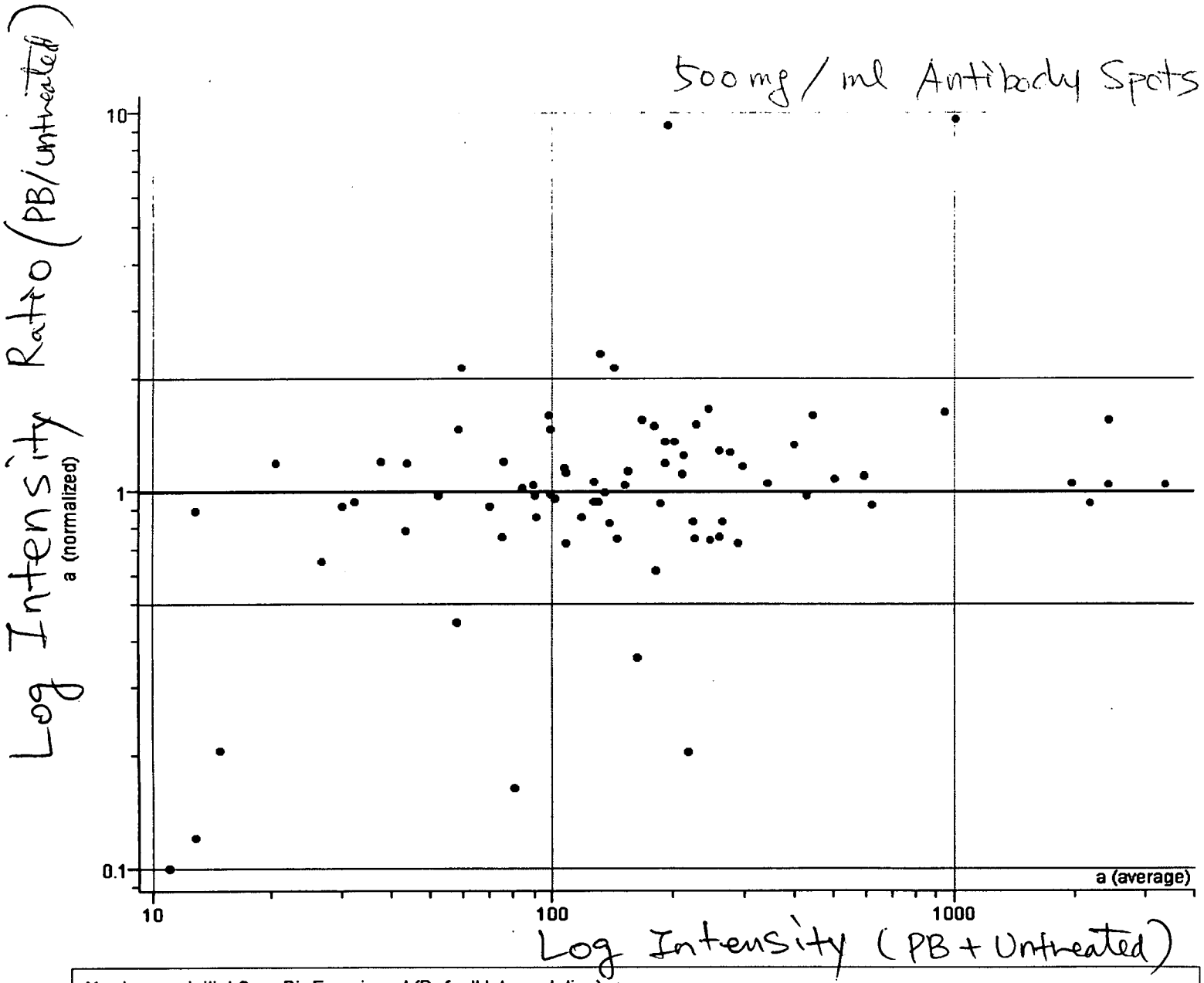
Fig. 7. Electronic data sheets for 4th antibody chip production (1st plate)



50 mg/ml Antibody Spots



X-axis: initial GenePix Experiment (Default Interpretation), a
Y-axis: initial GenePix Experiment (Default Interpretation), a
Gene List: 50 ug only (78), 15 genes selected, 12 selected genes not in list



X-axis:	initial GenePix Experiment (Default Interpretation), a
Y-axis:	initial GenePix Experiment (Default Interpretation), a
Gene List:	78 genes with 500 ug (78), 15 genes selected, 12 selected genes not in list

Microarray technology: Regulation of gene expression has been analyzed by a throughput method such as microarray technology which simultaneously monitors expression levels of thousands of genes. Automated chip construction, use of fluorescent signals and custom digital image analysis make it possible to monitor gene expression of thousands of genes and obtain expression profiles of environmental toxicants (1). However, mRNA expression of a gene may not correlate with protein expression because of differences in rates of mRNA translation/degradation and post-translational modification.

Proteomics: Mass spectrometry with and without prior separation of proteins by affinity chips or two-dimensional pulse-field gel eletrophoresis has been developed and is in use at a few laboratories. The drawbacks of this method is that it is labor-intensive and requires a high technical skill (2).

Protein and antibody microarray technology: Recently, protein and antibody microarrays have been developed using the robotic system used for cDNA microarrays at Dr Haab's and others' laboratories (3-6). Most microarray centers are already equipped with robotics to print proteins or antibodies on a modified glass slide, scanners and software required to visualize and quantitate fluor signals and bioinformatic tools for data mining. Moreover, the experimental procedure and data mining of protein and antibody array analyses are almost identical to the DNA arrays. Antibody microarray technology is extremely user-friendly. However, the collection of antibodies against all proteins expressed in cells is an almost impossible task. Moreover, development of functional and specific antibody arrays is challenging because limited monoclonal antibodies are available for microarray chip production. Most of the time, form-specific antibodies of interest differ greatly in their binding strength to the antigen. Targeted arrays with form-specific antibodies of closely related proteins involved in drug metabolism will be an invaluable tool, but this emerging technology has to be improved to accommodate the use of monoclonal, form-specific polyclonal and anti-peptide antibodies on one chip.

Drug-metabolizing enzymes and cytochrome P450 (CYP): There are Phase I and II drug-metabolizing enzymes. CYPs are Phase I enzymes which normally catalyze the first step of xenobiotic metabolism with a required co-enzyme, CYP-NADPH reductase or cytochrome b_5. Glutathione S-transferases, sulfo-transferases, epoxide dehydrolases, N-acetyl transferases and UDP-glucuronyl transferases are Phase II enzymes in drug metabolism. CYPs play an active role in the metabolism of numerous physiological substrates such as steroid hormones, fatty acids, prostaglandins and bile acids as well as countless xenobiotics including drugs, chemical carcinogens, insecticides, petroleum products, and other environmental pollutants. Whereas oxidative metabolism catalyzed by CYP can result in detoxification, in some instances, it results in metabolic activation of a chemical to cytotoxic and/or carcinogenic form. Although the liver is the primary organ for drug metabolism by a CYP, extra-hepatic tissues such as lung, kidney and intestine also play an important role for detoxification or biotransformation of xenobiotics. Each tissue has a unique pattern of CYP isozyme distribution and regulatory mechanism of CYPs. The P450s in the brain, pituitary grand and prostate (7-9) are especially of interest because of their possible physiological function and their potential role for cytotoxicity and/or carcinogenicity. The expression of CYPs are increased by xenobiotics as well as endogenous compounds such as fatty acids and steroids (10,11), developmentally regulated and induced by pathophysiological conditions such as starvation and diabetes (10-12). CYP protein expression is regulated by various mechanisms (10-12).

Differential regulation of CYP subfamilies: Our long-term goal is to produce or license-in antibodies against all human, rat and mouse CYP subfamilies and produce antibody arrays. CYP subfamilies are differentially regulated. The CYP3A subfamily which is the most abundant CYP subfamily in human and rat liver and intestine is a key contributor in the metabolism of a large number of therapeutic agents. CYP3A activity can be dramatically induced and/or potently inhibited by a diverse array of therapeutic agents. Five CYP3A forms, 3A1, 3A2, 3A9, 3A18 and 3A23, have been cloned and isolated from the rat. Three human CYP3A forms, 3A4, 3A5 and 3A7, have been cloned (13). The CYP3A forms have been shown to be differentially regulated in rat liver in response to physiological and xenobiotic stimuli (Table 1). CYP3A2, 3A9 and 3A18 are differentially expressed in juvenile and adult rats in a sex-dependent manner (14). Neonatal xenobiotic exposure can partially disrupt the sex-specific and developmental pattern of CYP3A expression (15). The various rat hepatic CYP3A forms are all inducible by xenobiotics, although which are the most efficacious xenobiotic inducers varies between forms (Table 1). For example, dexamethasone (DEX) strongly induces CYP3A1/23 and 3A18, induces CYP3A2 in males but not females,

and weakly induces CYP3A9 (14,16). Only CYP3A9 among CYP3A appears to be inducible by estrogen (17). Human CYP3A forms are also differentially regulated in regard to developmental stage and responsiveness to xenobiotics (18). In addition to liver, CYP3A has been reported to be expressed in the kidney, lung, breast, GI tract, leukocytes and brain (19-21). Renal CYP3A levels may be a key determinant in the development of spontaneous or drug-induced hypertension, possibly as a result of CYP3A corticosterone 6β-hydroxylase activity (22-24). The major renal CYP3A form is different from the major hepatic form in both humans and rats, suggesting that the function and regulation of renal and hepatic CYP3A may differ (19,25). Brain CYP3A is likely to be important in localized drug metabolism and, hence, may be a key determinant of concentrations of central nervous system-acting drugs such as imipramine in target cells (17).

Table 1. Expression of rat CYP3A with age, sex, tissue specificity and xenobiotic responsiveness.

CYP3A Form	Juvenile[a] M	Juvenile[a] F	Adult[a] M	Adult[a] F	Known Tissues	Efficacious Inducer[b]
3A1/23	-	-	-	-	kidney[c], liver	Dex
3A2	+	+	++	-	liver[c]	PB
3A9	-	-	?	++	brain[c], liver	Estrogen, PB
3A18	+	+	++	?	liver	Dex

[a] Levels in untreated animals.
[b] DEX, dexamethasone; PB, phenobarbital.
[c] Indicates that this form is a major CYP3A form in this tissue.

Antibody microarrays for drug-metabolizing enzymes: CYP unigene entries of 11 animal and 8 plant species, 2 lower eukaryotes and a bacteria are summarized and posted on Dr. Nelson's CYP homepage (http//drnelson.utmem.edu/ Cytochrome p450.html). About 30 and 50 rat and human unigene entries, respectively, are posted. Other enzymes involved with drug-metabolism are Phase II drug-metabolizing enzymes, co-enzymes of CYPs, carboxylesterases, and prostaglandin H_2 synthases (PGHSs, also called cyclooxygenases, COXs).

Antibody microarrays for drug-metabolizing enzymes are not available. Thus, **during Phase I**, we will develop antibody arrays for rat drug-metabolizing enzymes and proteins with related functions (in total, ~100 antibodies, see Table 2).

During Phase II study, we will produce antibody microarrays for human and mouse drug-metabolizing enzymes and proteins with related functions. During Phase II, for chip production, we will include additional antibodies for post-translationally modified proteins. We will also include antibodies for receptors involved with expression of drug-metabolizing enzymes, e.g., constitutive androstane receptor (CAR) and pregnane X receptor (PXR) which regulate expression of CYP2B and CYP3A, respectively. During Phase II, comprehensive antibodies and proteins for reference mixes for rat, human and mouse drug-metabolizing enzymes and proteins with related functions will be collected. The antibody microarrays can be used for drug and environmental toxicant screening. We will also produce web-based software for customers to use for identification of up- or down-regulated proteins which will be linked to NCBI UniGene databases for further information of the proteins.

Thomas et al. (26) reported 5 well-studied toxicological classes and a representing toxicant in each class. During Phase II study, we will attempt to produce a subarray which contains exposure markers of the 5 classes of toxicants. We will treat rats with 5 classes of toxicants and identify markers of exposure to the 5 classes of toxicants. Six markers of each class of toxicant will be selected to produce a diagnostic chips with ~30 (5 classes of toxicants X 6 markers) antibodies for drug-metabolizing enzymes and related proteins and 4 housekeeping genes.

Products that will arise from Phases I and II of this project are:

(i) Antibody microarray chips containing a comprehensive collection of drug-metabolizing enzymes and proteins with related functions
(ii) Web-based software produced from chip design files and one-step links to databases to facilitate interpretation of the microarray data
(iii) Reference proteins
(iv) Markers of exposure to 5 classes of toxicants in rats

(v) Diagnostic antibody microarray chips containing ~35 antibodies including antibodies for up- or down-regulated proteins by 5 classes of toxicants and house keeping genes
(vi) Additional antibodies and purified proteins
(vii) Contract services for drug and toxicant screening and custom subarray production.

2. Technical Objectives

During Phase I, we will (a) find the best condition for antibody chip production and produce rat drug-metabolizing enzyme chips, (b) produce rat reference proteins and (c) perform array analyses with rat liver samples to explore utility of the chips. The results obtained during Phase I study provide a solid foundation for Phase II study. Our long-term goal is to improve the antibody microarray technology and, then, produce rat, human and mouse drug-metabolizing enzyme antibody microarrays. In addition, to take advantage of proteomics, we will produce arrays for post-translationally modified proteins.

Products that will arise from Phases I and II of this project are (i) antibody microarray chips containing comprehensive collection of drug-metabolizing enzymes and proteins with related functions, (ii) web-based software and links to databases for customers to facilitate interpretation of the microarray image, (iii) reference proteins, (iv) markers of exposure to 5 classes of toxicants in rats including phenobarbital treatment, (v) diagnostic antibody microarray chips containing ~35 antibodies including antibodies for up- or down-regulated proteins by 5 classes of toxicants, (vi) additional antibodies and purified proteins, and (vii) contract services for drug and toxicant screening and custom subarray production.

Objectives of Phase I research include:

1. Rat drug-metabolizing enzyme antibody chip production: About 40 antibodies for drug-metabolizing enzymes have been characterized and are available at Detroit R&D (Table 2). Additionally, ~60 antibodies of interest will be obtained from commercial sources. Titer (binding strength) of the antibodies for purified proteins will be assayed by ELISA. Prior to antibody chip production with ~100 antibodies, test chips will be produced with ~10 antibodies (0.2, 0.5, 1, 2 and 4 ng of polyclonal and monoclonal antibodies). The test chip production is to study (a) what method is suitable for immobilizing antibodies on the slide to keep high binding activity of the antibodies, (b) whether spotting concentrations of antibodies can be calculated from their titers obtained by ELISA, and (c) sensitivity of the arrays (the detection range of a protein). Two internal controls will be developed to serve as landmarks for orientation of the chips and as internal controls for probe labeling efficiency.

2. Production of rat reference proteins: Reference proteins will be produced to make a comparison of the array data from different times and various laboratories. Reference proteins have to contain all proteins which are recognized by antibodies spotted on the chip. We will produce reference mixes by combining purified or partially purified antigens and by pooling hepatic microsomes + cytosol obtained from rats treated with various CYP inducers of Phases I and II drug-metabolizing enzymes. Both reference mixes will be used for microarray analyses as described in Objective 3.

3. Exploration of utility of the antibody chips: Antibody array analyses will be carried out as described previously by Haab et al. (3) with liver microsomes + cytosol obtained from rats after treatment with PB (100 mg/kg/day for 3 days) using a reference design. Antibody array analyses will also be carried out with whole cell extracts of rat liver primary cultured cells obtained after treatment with PB (1 mM). Control and PB-treated proteins will be labeled with both Cy-3 and Cy-5 for fluorescence swaps with reference mixes. Antibody arrays will be carried out with both native and denatured proteins. After hybridization, imaging will be carried out using GenePix 4000A (Axon Instruments) or Agilent G2565AA two-laser microarray scanner. Statistics and data mining of the arrays will be carried out using Rosetta Resolver microarray data analysis system, Agilent microarray data analysis software or GeneSpring microarray clustering and statistical analysis (Silicon Genetics). The GeneSpring software package will be used for several multiple test corrections, including Bonferroni, step-down, Westfall and Young permutation, and Benjamini and Hochberg FDR. We will use the Westfall and Young permutation test and the permutation method (SAM) developed in Dr. Tibshirani's biostatistics lab at Stanford University (30). Both methods account for the multiple testing problems. Data analysis files obtained using chip design files will contain identity of each protein. The results obtained by microarray analyses will be verified by ELISA.

3. Phase I Work Plan

Objective 1: Rat drug-metabolizing enzyme antibody chip production.

Rationale: Specificity of ~40 antibodies for drug-metabolizing enzymes available at Detroit R&D have been characterized at laboratories of Detroit R&D or our collaborators. Additionally, ~60 antibodies of interest will be obtained from various commercial sources (Table 2).

Prior to production of antibody chips with the ~100 antibodies, suitable chip production methodology will be developed by producing test chips with ~10 antibodies of CYP 3A, house keeping proteins and internal controls. We will compare antigen-binding capacity of the antibodies immobilized by two different methods and select a better method for spotting antibodies on the slide.

Theoretically, high affinity monoclonal and low affinity anti-peptide antibodies can be spotted on the same chip. The ratio of a specific protein expressed in control and experimental samples can be obtained from a weak affinity antibody on a chip. However, without printing a much higher concentration of the weak antibody on the chip, the data will not be reliable primarily due to a high background.

Table 2. A list of antibodies to print on chip for rat drug-metabolizing enzyme microarrays.

Antibody available at Detroit R&D	Commercially available	Antibody available at Detroit R&D	Commercially available
Anti-rat P450 antibody		**Anti-rat Phase II drug metabolizing antibody**	
1A1		N-acetyltransferase form-I	
1A1/ 1A2		N-acetyltransferase form-II	
2A		GST-alpha	
3A1		GST-mu	
3A2		GST-pi	
3A23		microsomal epoxide hydrolase	
3A1/3A2/3A23[b]			UDP-glucuronosyltransferase
	1B1	sulfotranferases	
2B1		**Antioxidant antibody**	
2B1/2B2		Superoxide Dismutase	
2B1/2B2/2B3		Catalase	
	2C6		Glutathione peroxidase
2C7[a]			Glutathione reductase
2C11			r-Glutamylcysteine Synthase
	2C13	**Anti House Keeping Protein Antibody**	
2C23			Tubulinn
2C[c]			beta-Actin
	2D[c]		G3PDH
2E1			alpha-Actin
2G1			IRP-1
2J3/2J4			Malate Dehydrogenase
Co-enzyme for CYP activity antibody			citrate synthase
	Cytochrome b5[a]	**Anti oxidative stress antibody**	
P450 -NADPH-Reductase			iNO Synthase
Carboxylesterase			nNOSynthase
	sPLA2		eNOSynthase
	Flavo-mono-oxygenase 1[c]	COX-1	
	Flavo-mono-oxygenase 2[c]	COX-2	
	Flavo-mono-oxygenase 3[c]	nitrotyrosine	
	Flavo-mono-oxygenase 4[c]		5-Lipoxygenase
Antibodies to Mitochondria-Related Proteins			Thromboxane Synthase
	Bax		prostaglandinE Synthase
	Bcl-2	Calpain	
Cytochrome C			NFkappaB
	PBR		PPAR (peroxisome Proliferator-activated
Apoptosis-related antibodies			receptors)-Alpha
	Apaf1		P-Glycoprotein (P-gp)
	Bad		TNF-alpha
	Bak		Heme Oxygenase-1
	BID		Heme Oxygenase-2
	Caspase3		Guanylyl Cyclase α1
	Caspase8		Guanylyl Cyclase β1
	Caspase10		Beta catenin
	FADD		CEA(Carcinoembryonic antigen)
	Fas		Antioxidant-like protein 1
	p53		
	PARP		
	SURVIVIN		
	TNFR2		
	TARDD		
	TRAIL		
	Rb		
Intracellular Protein Degradation antibodies			
	Calpastatin		
	E1 Ubiquitin		
	Ubiquitin		

a: Dr. H. Kim successfully produced antibody against this CYP at other institutes
b: Human CYP3A4 which is specific for rat CYP3A subfamily
c: Polyclonal antibody produced against human form but most likely cross reacts with rat form

Our hypothesis is that, when monoclonal antibodies with similar affinity to the antigens are not available, weak binding antibodies, e.g., anti-peptide antibodies, can be successfully used by spotting a higher amount of antibodies. Fold induction of the protein expression obtained by the array analyses will be compared with results by ELISA. If our hypothesis is proven that the titer of an antibody can be used to calculate the spotting amount on a slide, we will produce drug-metabolizing enzyme microarray chips with ~100 antibodies of interest listed in Table 2.

Experimental Approach: Antibody array chips will be produced at Dr. Dombkowski's laboratory at the Center for Environmental Health Sciences, Wayne State University.

(a) Test chip production with ~10 antibodies and hybridization:

(i) Titer (binding strength) of a polyclonal antibody for CYP3A1/2/23, two monoclonal antibodies for CYP3A1 and CYP3A2 and a polyclonal anti-peptide antibody for CYP3A23: The titer of the antibodies will be determined by an ELISA with each well coated with non-denatured (native) and denatured rat CYP3A proteins obtained by immunoaffinity chromatography and purified rat tubulin, β–actin and gryceraldehyde 3-phosphate dehydrogenase (G3PDH) (Sigma Co.). Denatured samples will be obtained after heat-denaturation (98°C for 2 min).

(ii) Antibody immobilization methods: Two different antibody immobilization methods (poly-L-lysine coated slides with photo-reactive cross-linking layer and superaldehyde-modified glass slides) will be used to compare coating efficiency and protein binding activity of the immobilized antibodies.

(iii) Antibodies on the test chip: The test chips will contain 0.2, 0.5, 1, 2 and 4 ng of (i) 4 antibodies against CYP3A family, (ii) 3 house keeping proteins (tubulin, β-actin and G3PDH), and (iii) two internal controls for chip orientation and probe labeling efficiency. Array analyses will be carried out with a polyclonal antibody for CYP3A1/2/23, two monoclonal antibodies for CYP3A1 and CYP3A2 and a polyclonal anti-peptide antibody for CYP3A23 printed on the chips to verify our hypothesis that the high background problem of a weak binding antibody can be corrected by spotting a higher amount of antibodies on the chip.

(iv) Landmarks and internal controls for probe labeling efficiency: Two internal controls will serve as landmarks for orientation of the chips and as an internal control for probe labeling efficiency. Two candidates to be tried with this test chip are (a) Met + FLAG peptide and (b) poly-His (6-His) which are recognized by anti-FLAG and anti-6-His, respectively. These peptide sequences to be used for fluorescent signal normalization and landmarks are not present in mammalian cells.

Antibodies for FLAG peptides and 6-His are available from Sigma Co. (Cat. #F4042) and Covance Research Products, Inc. (Cat. #PRB-156C), respectively. FLAG peptides are available from Sigma Co. but Met-FLAG fusion peptides, which are recognized by anti-FLAG, are not available. Met-FLAG peptides + C-terminal Cys (Met-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-Cys) and 6-His peptides will be synthesized and cross-linked to BSA by sulfhydryl side chain of Cys and C-terminal carboxyl group of His, respectively, with BSA. A known amount (30 ng) of the landmark peptide conjugates will be added to proteins (30 μg) for both Cy-3 and Cy-5 labeling and used for determination of probe labeling efficiency or signal normalization. A normalization factor (NF) obtained by this method will be compared with an NF obtained from total Cy-3 or Cy-5 bound to antibodies on the chip.

(v) Spotting antibodies on chips: Antibodies (0.2, 0.5, 1, 2 and 4 ng) will be printed on two different kinds of glass slides in triplicate using a GeneMachines Omnigrid (see an attached letter from Dr. Landgraf at the Genomics core, Michigan State University, for use of the spotting robot). Spotting levels of high affinity antibodies will be ~0.5 μg/μl, 1 nl per spot. Spotting of 0.1-1 ng of monoclonal antibody has been reported to be satisfactory (3,6). The antibodies will be transferred into a 384-well plate and spotted onto multiple slides. The arrays will be spotted with solid pin technology with sufficient washing between spotting to eliminate cross contamination. A limited number of pins will be used to spot the arrays to decrease the effect of pin variation. Humidity will be maintained at a sufficient range to produce spot size between 100-200 microns.

(vi) Coating efficiency and background levels of the slide: Coating efficiecy and retention of the antibodies on the chip will be investigated with both poly-L-lysine coated and superaldehyde-modified glass

slides by hybridization of the chips with a mixture of Cy-3 labeled anti-rabbit IgG and Cy-5-labeled anti-mouse IgG with 0.1% BSA and 0.01% Tween-20. Cy-5-labeled anti-mouse IgG will bind monoclonal antibodies for CYP3A1, CYP3A2, tubulin, β-actin and G3PDH. Cy-3-labeled anti-rabbit IgG will bind polyclonal antibodies for CYP3A and CYP3A23. Two landmarks, Met-FLAG peptides-Cys conjugated BSA and 6-His peptide conjugated BSA (30 ng) will be added to the IgG (30 μg) prior to labeling with the fluors. Fluorescent signals will be determined with both poly-L-lysine coated and superaldehyde-modified slides and the coating efficiency and non-specific binding will be analyzed.

(vii) Sensitivity (detection range) of antibody chips: 0.1, 0.5 or 2 ng of purified tubulin, β-actin and G3PDH will be mixed, cross-linked with Cy3 and Cy5 and purified. Three separate hybridizations will be carried out with 0.1% BSA and 0.01% Tween-20 and Cy3 (green) and Cy5 (red) signals will be obtained. Median $\log_{10}$ (red signal/green signal) will be plotted against $\log_{10}$ antigen concentrations (0.1, 0.5 or 2 ng) or $\log_{10}$ antibody concentrations (0.2, 0.5, 1, 2 and 4 ng). Straight diagonal lines are expected when antigen levels are in the detection range as previously described by Haab et al. (3).

(viii) Hybridization of the antibodies with samples by reference design: Blocking of antibody chips overnight at 4°C with 3% non-fat milk/PBS/0.02% sodium azide blocking solution will be carried out as described by Haab et al. (3).

Two landmarks, Met-FLAG peptides-Cys conjugated BSA and 6-His peptide conjugated BSA (30 ng) will be added to reference mixes (10 ng), microsomes + cytosol obtained from untreated male rats cytosol (30 μg, 0.2-2 mg/ml solution) and microsomes + cytosol obtained from male rats after PB treatment cytosol (30 μg). The mixtures will be labeled as previously described (3,6) with NHS-ester activated Cy-3 and Cy-5 (Amersham Biotech, PA23001, PA25001), purified and concentrated using microconcentrator spin columns (Amicon Nicrocone 10, a 10 kDa molecular weight cutoff) as previously described (3). Cy-3 and Cy-5 incorporation will be determined using a fluorometer. Fluorescent-tag swapping will be carried out. Hybridization (in total, 4 hybridizations) and washing will be carried out as previously described (3,6) with 0.1% BSA and 0.01% Tween-20.

(ix) Imaging and quantitation: After hybridization, the chip will be dried and imaging will be carried out using GenePix 4000A (Axon Instruments, Inc., Foster city, CA) or Agilent G2565AA two-laser microarray scanner. Normalization of expression levels (Cy-3/Cy-5 or Cy-5/Cy3) of CYP3A (all in 3A subfamily), CYP3A1, CYP3A2 or CYP3A23 will be carried out using levels of both landmark signals and total Cy-3 or total Cy-5 signal. A signal of each spot will be calculated using a median value but not a mean value because, with our previous experience with *Xenopus* chip production (SBIR contract Phase I), the median value was found to be a better index of the signal.

(x) Verification of microarray analysis: Fold induction of CYP3A (all in 3A subfamily), CYP3A1, CYP3A2 or CYP3A23 after PB treatment obtained by antibody array analyses will be compared with results obtained by Western blot analysis (denaturing condition) and ELISA (non-denaturing condition) of the control and PB treated microsomes.

(xi) Stability of antibodies spotted on chip: Antibody chips will be washed and dried as previously described by Haab et al. (3) and refrigerated in a dessicator. Antibodies are stable and usually their active sites are not easily damaged. Chips produced during Phase I are for an immediate use. However, for marketing, stability or a shelf-life of the antibody chip is a factor for a successful commercialization. During Phase II study, we will study stability of the antibody chips.

(b) Antibody chip production with ~100 antibodies:

(i) Selection of a suitable immobilization method: Antibody binding efficiency and sensitivity of the two different chips will be compared to select a better method between the two immobilization methods.

(ii) Verification of our hypothesis that the high background problem of a weak binding antibody can be corrected by spotting a higher amount of antibodies on the chip: Results obtained by array analyses of CYP3A and house keeping proteins will be compared with results from ELISA.

(iii) Titer of additional antibodies: If array analyses with the test chips prove that ELISA can be used to predict the outcome with array analyses, titers of ~100 antibodies will be determined by an ELISA with each well coated with non-denatured (native) and denatured reference proteins.

(iv) Rat drug-metabolizing enzyme antibody chip production: The amount of antibodies to be spotted on the chip will be decided after our hypothesis is proven to be correct by the test array analyses. It is expected that the amount of a majority of anti-peptide antibodies on the chip will be ~4 fold higher than the amount of monoclonal antibodies because of their weaker binding to the protein antigen. The antibodies listed in Table 2 will be spotted in 10 repeating blocks. Antibodies against landmarks and internal controls (Met-FLAG peptides-Cys-conjugated BSA or 6-His peptide-conjugated BSA) will be spotted on the left bottom corner (A1 position) of each block. All spotting space surrounding the landmarks will be empty for fast visualization and identification of orientation of each block.

Anticipated results and potential problems:

Recently, Drs. Kim and Kaplan at Detroit R&D successfully produced *Xenopus* cDNA microarray chips during Phase I study of an NIEHS contract. Antibody microarray chips will be produced using a spotter, a scanner, fluors (Cy-3 and Cy-5) and data mining software used for the *Xenopus* cDNA microarrays.

Optimal conditions for antibody chip production will be studied at Dr. Dombkowski's laboratory with Dr. Haab's assistance. Dr. Haab at Van Andel Institute, Grand Rapids, pioneered antibody microarray production and analyses at Dr. Brown's laboratory at Stanford University (3). He will serve as a consultant for this project. Dr. Dombkowski has extensive experience in microarray analysis and Ms. Diakiw produced antibody microarray chips when she was employed by Genomic Solutions. Thus, functional, specific antibody chips are expected to be produced.

A few antibody microarray chips are commercially available. They are made of monoclonal antibodies. Targeted antibody arrays for drug-metabolizing enzymes are not available. Detroit R&D is marketing a few monoclonal antibodies for rat CYPs. However, antibodies for some of the CYP subfamily were produced by anti-peptide antibody technology using synthetic peptide immunogens. Some polyclonal antibodies for rat CYPs recognize all CYPs in the subfamily but are still specific for the subfamily. As far as the polyclonal antibodies are specific for the subfamily, it will be included in the chip production. Anti-peptide antibodies are specific but bind somewhat weakly. Increasing spotting concentrations of the weak antibodies may correct high background. Some polyclonal antibodies will compete for monoclonal antibodies for one of the subfamily, e.g., polyclonal antibodies for CYP3A will compete with monoclonal antibodies against CYP3A1 for CYP3A1 proteins in hybridization solution.

Objective 2: Production of rat reference proteins.

Rationale: Reference protein mixes will be produced by (a) mixing purified proteins and (b) pooling hepatic proteins obtained from male rats after treatment with various inducers of drug-metabolizing enzymes. Array experimental designs with two different reference mixes are described in Objective 3. Use of the reference design will make it possible to compare array data obtained at different times or at other laboratories.

Experimental Approach:

(a) Reference proteins produced by mixing purified or partially purified proteins:

(i) Reference proteins for antibodies available at Detroit R&D (Table 2): The proteins or peptides used for antibody production will be available for this project. Rat CYP1A, CYP3A, CYP2C and CYP2E subfamilies will be purified by immunoaffinity chromatography of rat microsomal proteins using polyclonal antibodies. Synthetic peptides and molecules will be cross-linked to BSA to be used as reference proteins. Lys residues of BSA are needed for the fluorescent probe labeling and a molecular mass of probes has to be bigger than the 10 kDa (purification of probes will be carried out with a 10 kDa molecular cut-off membrane).

(ii) Reference proteins for commercially available antibodies (Table 2): Most of the proteins or molecules are commercially available. A few antibodies are made from synthetic peptides or chemical antigens. We will obtain synthetic peptides or chemicals and cross-link them to BSA.

(b) Reference proteins produced by pooling hepatic proteins obtained from rats after treatment with various inducers: Hepatic whole proteins, microsomes or cytosol will be isolated as previously described (27,28) from male Sprague-Dawley rats treated with PB (100 mg/kg/day for 3 days), pyridine (200 mg/kg/day for 3 days), DEX (10 mg/kg/day for 4 days), 3-methycholanthrene

(single injection of 25 mg/kg) and clofibrate (200 mg/kg/day for 3 days). Whole proteins, microsomes or cytosol from various inducers will be pooled. For primary cultured cells, a whole cell extract will be used for hybridization analysis. However, for rat tissue samples which can be obtained in a gram level, it is beneficial to carry out antibody microarray analysis with microsomes + cytosol. Thus, we will prepare reference proteins with (i) total whole cell reference proteins for use with primary cultured cell microarrays, and (ii) microsomes + cytosol reference proteins for *in vivo* studies.

Anticipated results and potential problems:

Use of reference proteins for antibody microarrays is advantageous for an inter-laboratory comparison of the array results. However, unless the same reference mix is used, a comparison of the data is misleading. Though reference mixes can be produced in large quantity and stored as an aliquot at -80°C, the protein concentration can be changed during storage. Thus, the protein mix will be stored at -80°C in a single use portion after lyophilization.

Objective 3: Exploration of utility of the antibody chips.

Rationale: Antibody array analyses will be carried out with liver microsomes + cytosol obtained from rats after treatment with PB (100 mg/kg/day for 3 days) using a reference design. Antibody array analyses will also be carried out with whole cell extracts of rat liver primary cultured cells obtained after treatment with PB (1 mM). Control and PB-treated protein samples will be labeled with both Cy-3 and Cy-5 to be used for fluor swapping. Hybridization with antibody chips will be carried out with native and denatured proteins. Reference mixes will be hybridized with control or PB-treated samples.

Experimental Approach:

(a) Microsomes + cytosol isolated from liver tissue obtained after treatment of rat with PB:

Landmarks and internal controls for probe labeling efficiency, Met-FLAG peptides-Cys conjugated BSA or 6 His peptide conjugated BSA (30 ng) will be added to purified protein reference mix (50 ng: 0.5 ng/protein x 100 proteins) or a liver reference mix (30 μg, 0.2-2 mg/ml solution) (see Objective 2). The internal control will be added to liver microsomes + cytosol (30 μg, 0.2-2 mg/ml solution) obtained from control rats (saline-treated) and rats after PB treatment produced by mixing microsomal and cytosolic proteins by 1:1.

The proteins will be labeled with Cy-3 and Cy-5. Most antibodies will recognize both native and denatured proteins. However, some of the antibodies prefer native or denatured proteins. Thus, antibody array analyses will be carried out with both native and denatured proteins. Denatured proteins will be produced by keeping at 98°C for 2 min after labeling with Cy-3 or Cy-5. Concentration and purification of the probes will be carried out using microconcentrator spin columns (Amicon, 10 kDa molecular weight cuttoff) as described in Objective 1. The probes made from microsomes + cytosol will contain a majority of drug-metabolizing enzymes. Hybridization analyses will be carried out with 0.1% BSA and 0.01% Tween-20 using a chip containing 10 repeating spots of an antibody as previously described (3,6) (see Objective 1). Hybridization analyses with native and denatured microsomes + cytosol probes will be carried out with both purified protein mixes and pooled liver microsomal and cytosolic protein references as follows:

1. Cy-3 labeled reference mix vs. Cy-5 labeled **control** microsomes + cytosol
2. Cy-5 labeled reference mix vs. Cy-3 labeled **control** microsomes + cytosol
3. Cy-3 labeled reference mix vs. Cy-5 labeled **PB** microsomes + cytosol
4. Cy-5 labeled reference mix vs. Cy-3 labeled **PB** microsomes + cytosol

(b) Whole cell extracts of rat liver primary cultured cells obtained after treatment with PB (1 mM):

Whole cell extracts will be used for array analyses of rat primary cultured hepatocytes because isolation of microsomes and cytosol will cause protein loss. Results obtained from rat liver tissues (Objective 3, a) will reveal whether native or denatured proteins have to be used for drug-metabolizing enzyme chips. Hybridization analyses with both purified protein mixes and pooled liver whole cell extract protein references will be carried out as follows:

1. Cy-3 labeled reference mix vs. Cy-5 labeled **control** total cell extract
2. Cy-5 labeled reference mix vs. Cy-3 labeled **control** total cell extract
3. Cy-3 labeled reference mix vs. Cy-5 labeled **PB** total cell extract
4. Cy-5 labeled reference mix vs. Cy-3 labeled **PB** total cell extract

(c) Identification of up- or down-regulated proteins: After hybridization, images will be obtained in a gray scale by scanning the chips at 532 nm (green) or 635 nm (red) and the gray scale images will be false-colored in green and red, respectively. The pseudo-colored images will be combined to produce a composite image. In the composite image, when equal amount of Cy-3- and Cy5-tagged probes are bound, color of the spot is yellow. Imaging will be carried out using GenePix 4000A (Axon Instruments) or Agilent G2565AA two-laser microarray scanner. Antibodies for anti-Met-FLAG peptides or anti-6-His peptide will be spotted as a landmark at the A1 position of each block (each block is made of 1 to 10 for the first dimension and A to J for the second dimension). The images obtained without correction of the gray scale images to compensate for differences in labeling efficiency of Cy3 and Cy5 will be corrected by multiplying with a normalization factor.

(d) Bioinformatics: Statistics and data mining of the arrays will be carried out at Detroit R&D with assistance of Dr. Dombkowski using Rosetta Resolver microarray data analysis system, Agilent microarray data analysis software or GeneSpring microarray clustering and statistical analysis (Silicon Genetics). Spreadsheets containing identity of each protein and it's location in the 384-well plate will be produced with design files. The GeneSpring software package will be used for several multiple test corrections, including Bonferroni, step-down, Westfall and Young permutation, and Benjamini and Hochberg FDR. Standard ANOVA analysis assumes that the distribution of experimental errors is normal, and recent publications indicate that this is not a safe assumption with the microarray technology (29). Permutation-based methods do not depend on distribution normality, thus are preferred where data normality cannot be assumed. Our large number of replicated experiments will be well complemented by permutation-based statistical methods. We will use the Westfall and Young permutation test and the permutation method (SAM) developed in Dr. Tibshirani's biostatistics lab at Stanford University (30). Both methods account for the multiple testing problems.

(e) Validation of the microarray analysis by ELISA: After identification of up- or down-regulated proteins after PB treatment, the results will be verified by ELISA.

Anticipated results and potential problems:

Differential expression of each drug-metabolizing enzyme with and without PB treatment will be obtained. Addition of internal controls for probe labeling efficiency will make data interpretation easier. The ~100 antibody collection still does not contain all antibodies which might play a role in drug metabolism. We will produce additional antibodies for drug-metabolizing enzymes using phage display, monoclonal antibody and anti-peptide antibody technologies during Phase II. We predict that expression of CYP2B and CYP3A subfamily will dramatically increase after treatment of rats or hepatocytes with PB.

Denatured proteins aggregate and may not be dissolved in 0.01% Tween. We will improve solubility of the protein by cleavage with cyanogen bromide which cleaves the carboxyl terminal of Met. Because Met is a rare amino acid in polypeptides, a chance of destroying an antigenic site of the denatured protein is low. Microarray analyses with the cleaved proteins will reveal whether the cleaved peptide with antigenic sites are fluorescently labeled.

Literature Cited

1. Nuwaysir, E.F., Bittner, M., Trent, J., Barrett, J.C., Afshari, C. A. Mol. Carcinogen. 24,153-159 (1999).
2. Tomlinson, I. M. and Holt, L. J. Genome Biol. 2(2), reviews 1004 (2001) and references there in.
3. Haab, B. B., Dunham, M. J., Brown, P. O. Genome Biol. 2, research 0004.1-0004.12 (2001).
4. Leukling, A., Horn, M., Eickhoff, H., Bussow, K., Lehrach H. et al. Anal. Biochem. 270, 103-111 (1999).
5. MacBeath, G. and Schreiber, S. L. Science 289, 1760-1763 (2000).
6. Streekumar, A., Nyati, M. K., Varambally, S., Barrette, T. R., Ghosh, D., Lawrence, T. S. and Chinnaiyan, A. M. Cancer Res. 61, 7585-7593 (2001).
7. Sundin, M., Warner, M., Haaparanta, T., and Gustafsson, J. J. Biol. Chem. 262, 12293-12297 (1987).
8. Warner, M., Strömstedt, M., Möller, L., and Gustafsson, J.-Å. Endocrinology 124, 2699-2706 (1989).

9. Warner, M., Tollet, P., Strömstedt, M., Carlström, K., and Gustafsson, J. Endocrinol. 122, 341 (1988).
10. Gonzalez, F. J. Pharmacol. Reviews 40, 243 (1989).
11. Gonzalez, F. J. Pharmac. Ther. 45, 1-38 (1990).
12. Porter, T. D., and Coon, M. J. J. Biol. Chem. 266, 13469-13472 (1991).
13. Nelson, D.R., Koymans, L., Kamataki, T., Stegeman, J.J., Feyereisen, R., Waxman, D.J., Waterman, M.R., Gotoh, O., Coon, M.J., Estabrook, R.W., Gunsalus, I. et al. Pharmacogenetics 6, 1-42. .(1996).
14. Mahnke, A., Strotkamp, D., Roos, P. H., et al. Arch. Biochem. Biophys. 337, 62-68 (1997).
15. Zangar, R.C., Springer, D.L. and Buhler, D.R. J. Toxicol. Environ. Health 38, 43-55 (1993).
16. Kim. E.Y., Krymgold, S., Putt, D.A. and Kim, H. FASEB J. 11, A818 (1997).
17 Wang, H.M. and Strobel, H.W. Arch. Biochem. Biophys. 344, 365-372 (1997).
18. Guengerich, F. P. in Cytochrome P450 (ed. Ortiz de Montellano.) Plenum Press, NY, 473-535 (1995).
19. Debri, K., Boobis, A.R., Davies, D.S. and Edwards, R.J. Biochem. Pharmacol. 50, 2047-2056 (1995).
20. Mahnke, A., Roos, P.H., Hanstein, W.G. and Chabot, G. Biochem. Pharmacol. 51, 1579-1582 (1996).
21. Wang, H.M., Kwashima, H. and Strobel, H.W. Biochem. Biophys. Res. Comm. 221, 157-162 (1996).
22. Watlington, C.O., Kramer, L.B., Schuetz, E.G., Zilai, J. et al. Amer. J. Physiol. 262, F927-931 (1992).
23. Basu, A.K., Ghosh, S., Mohanty, P.K. and Watlington, C.O. Hypertension 24, 480-485 (1994).
24. Ghosh, S.S., Basu, A.K., Ghosh, S., Hagley, R. et al. Biochem. Pharmacol. 29, 49-54 (1995).
25. Haehner, B.D., Gorski, J.C., Vanderbranden, M. et al. Mol. Pharmacol. 50, 52-59 (1996).
26. Thomas, R.S., rank. D.R., Penn, S.G., Zastrow, G.M., Hayes, K.R., pande, K., Glover, E., Silander, T., Craven, M.W., Reddy, J.K., Jovanovich, S.B., and Bradfield, C.A. Identification of toxicologically predictive gene sets using cDNA microarrays. Mol. Pharm. 60:1189-1194 (2001).
27. Kaul, K. L., and Novak, R. F. J. Pharmacol. Exp. Ther. 243, 384-390 (1987).
28. Kim, H., Putt, D., Reddy, S. Hollenberg, P. F., Novak, R. Pharmacol. Exp. Ther. 267, 927-936. (1993).
29. Kerr, M. K. and Churchill, G. A. Proc. Natl. Acad. Sci. USA, July 31; 98(16): 8961-8965 (2001).
30. Tusher, V. G., Tibshirani, R. and Chu, G. Proc. Natl. Acad. Sci. USA, 98(9), 5116-5121 (2001).

4. Related Research or R&D

Key personnel involved for this project are experts in antibody microarray chip production (Dr. Haab), diagnostic technology development including microarray analysis development (Drs. Kim, Dombkowski, Haab and Kaplan), Molecular Toxicology and drug metabolism (Drs. Kim and Novak) and non-radioactive probes development (Dr. Kim).

Hyesook Kim, Ph.D., Principal Investigator, has extensive experience in metabolism of xenobiotics and EIA and microarray development. Under Phase I and II SBIR, Dr. Kim characterized recombinant human CYPs, cloned rat PGD_2 synthase (GenBank accession number, AF021882), expressed human PG H_2 synthase form-2 (COX-2) in baculovirus/insect cell and human cell system, developed a DNA damage screening system and monoclonal antibodies against human PG H_2 synthase form-2 and eicosanoids cross-linked to carrier proteins. Since founding Detroit R&D, she was awarded an NIEHS Phase II contract and an NIEHS Phase II grant after successful completion of Phase I for each project. Recently, she finished an NIEHS Phase I contract project to develop a *Xenopus* microarray analysis. Dr. Kim successfully developed a methodology to produce form-specific, inhibitory anti-peptide antibodies against cytochromes P450 (US patent #5,866,688, 2/2/99), developed ELISAs for 8-epi-$PGF_{2\alpha}$ (US patents #5,891,622, 4/6/99) and ELISAs against arachidonic acid epoxygenase metabolites (US patent #6,440,682 and 6.534,282, 2 divisional patents issued on 8/27/02 & 3/18/2003, respectively). Patents on assessment of oxidant stress and detection and identification of toxicants by *Xenopus* gene expression profile are pending. She developed and successfully marketed over 100 products at Oxford Biomedical Research (1993-1997) and over 100 products at Detroit R&D (since 1998).

David J. Kaplan,Ph.D., Co-Investigator, has extensive experience in Molecular Toxicology including gene cloning, nucleotide sequencing, cell culture and gel shift assays. Recently, he successfully carried out *Xenopus* microarray analyses sponsored by NIEHS SBIR Phase I contract.

Alan A. Dombkowski, Ph.D., Subcontractor and Consultant, Director of Microarray and Bioinformatics Facility Core, Center for Environmental Health Sciences in Molecular and Cellular Toxicology with Human Applications and Assistant Professor at Wayne State University, is an expert in microarray development and Bioinformatics. Antibody microarray chips will be produced at his laboratory. He will assist Dr. Kim in the area of hybridization, scanning of microarrays and data mining. A letter attesting to his willingness to serve as a consultant and subcontractor for this project is appended to the application.

Amy Diakiw, at the Microarray and Bioinformatics Facility Core, Center for Environmental Health Sciences produced antibody microarrays at Genomic solutions and is working on a microarray project.

Brian B. Haab, Ph.D., at Van Andel Institute, has extensive experience in microarray analysis. He pioneered antibody array production. He will assist Drs. Kim and Dombkowski as needed in antibody chip production.

Raymond F. Novak, Ph.D., Director, Institutes of Environmental Health Sciences. and Director, the EHS Center in Mol. Cell. Toxicol. with Human Applications, Wayne State Univ., Consultant, is an expert in Environmental Toxicology, especially in studies of xenobiotics. He will assist Dr. Kim as needed in a xenobiotic treatment study.

5. Relationship with Future R&D

(a) Results expected from Phase I: Antibody microarray technology will be developed to produce arrays for drug-metabolizing enzymes. Utility of the antibody chips will be explored by analysis of up-regulated or repressed protein expression in rat liver and primary cultured hepatocytes after treatment with PB.

(b) The significance of the Phase I effort for Phase II: Our long-term goal is to improve antibody microarray technology and, then, produce rat, human and mouse drug-metabolizing enzyme antibody microarrays. In addition, to take advantage of proteomics, we will produce arrays for post-translationally modified proteins. Rat antibody microarray chip will contain only ~100 antibodies. However, the methods developed for antibody microarry analysis in Phase I will be utilized in Phase II to produce a human and mouse microarrays. Sample preparation, scanning and data interpretation skills developed during Phase I will be utilized in Phase II study to characterize the expression profiles of drug-metabolizing enzymes after chemical insults. Moreover, the results obtained in Phase I will be used to predict outcome of Phase II and develop a better strategy for microarray analyses and chip production. Thus, the results obtained during Phase I study will provide a solid foundation for Phase II study.

6. Potential Commercial Applications

Availability of a facile antibody microarray technology which simultaneously screens differential expression of drug-metabolizing enzymes and functionally related proteins will make an impact in the area of environmental toxicology. Commercially available antibody microarray chips which are subarrayed and characterized will be an invaluable tool for Toxicoproteomics.

Web-based distribution of chip design files and data for the antibody arrays and one-step links to UniGene databases of proteins will facilitate data interpretation by customers.

One drawback of this powerful technology is that scanning of chips after hybridization requires use of a fluorescence laser scanner. However, most universities and drug developing companies are already equipped or can afford to lease the scanner. One of the commercial products which Detroit R&D, Inc. will develop during the Phase I and II contracts will be the establishment of an affordable fee for service for drug-metabolizing enzyme antibody microarray technology. The microarray analysis, reference mixes and landmarks developed by Detroit R&D will facilitate identification of sensitive markers of environmental toxicants and facilitate drug discovery. Potential use by the Federal Government of the antibody microarrays is for screening and classifying environmental toxicants according to their mode of action and severity. This database will be used as a tool to decide permission levels of factory by-products. The arrays can also be used to investigate harmful effects of natural supplements and food additives in the market.

Products that will arise from Phases I and II of this project are (i) antibody microarray chips containing comprehensive collection of drug-metabolizing enzymes and proteins with related functions, (ii) web-based software and links to databases for customers to facilitate interpretation of the microarray image, (iii) reference proteins, (iv) markers of exposure to 5 classes of toxicants in rats including phenobarbital treatment, (v) diagnostic antibody microarray chips containing ~35 antibodies including antibodies for up- or down-regulated proteins by 5 classes of toxicants, (vi) additional antibodies and purified proteins, and (vii) contract services for drug and toxicant screening and custom subarray production.

7. Key Personnel and Bibliography: See following pages.